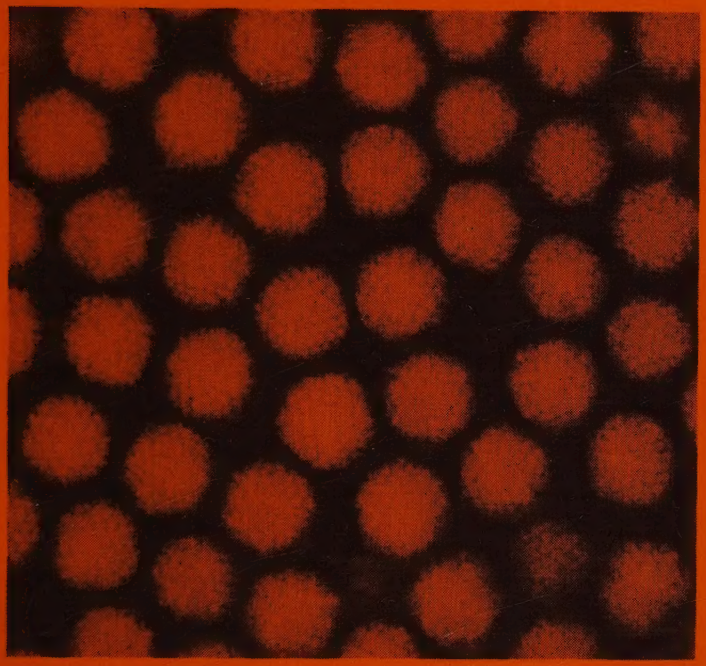
Neutralization of adenovirus



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Lund 1986

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by

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Cover shows adenovirus type 2

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PAPERS I-IV

This thesis is based on the following publications and manuscripts, which will be referred to in the text by their Roman numerals.

I. Wohlfart, C. and E. Everitt. Human adenovirus 2 as immunogen in rabbits yields antisera with high titers of antibodies against the nonstructural 72K DNA-binding protein. Virus Research 3:77-85 (1985).

II. Wohlfart, C. and E. Everitt. Assessment of specific virus infectivity and virus neutralization by a progeny virus immunotitration method as exemplified in an adenovirus system. Journal of Virological Methods 11:241-251 (1985).

III. Wohlfart, C., U. Svensson and E. Everitt. Interaction between HeLa cells and adenovirus type 2 virions neutralized by different antisera. Journal of Virology 56:896-903 (1985).

IV. Wohlfart, C. Neutralization of adenoviruses: Kinetics and stoichiometry. Submitted to Journal of Virology.

Immunizations of rabbits with human adenovirus 2 (Ad2) virions yield antisera with antibodies against most of the structural proteins of the virion together with two non-structural proteins - the 100K and the 72K proteins. The occurrence of antibodies against the latter protein was found to be due to a partial replication of Ad2 in the rabbits.

For the determination of the extent of neutralization of virus infectivity a progeny virus immunotitration assay was developed. Partially neutralized virions are added to HeLa cells in suspension cultures. After proper incubation the total amount of progeny virus produced in each sample is isolated, disrupted and quantified by rocket immunoelectrophoresis based on the hexon content. Neutralization is expressed as percent reduction in total virus yield in the sample as compared with control infections. The coefficient of variation of the assay for the determination of a partially neutralized virus sample was 5%.

Virions attached to HeLa cells at $4^{o}C$ are not neutralizable by an antifiber serum, which suggests that antifiber antibodies neutralize virions by mere aggregation. Anti-penton base serum caused a maximum of 50% neutralization. Virions, which were neutralized by either an anti-penton base or an antihexon serum,were trapped within intracellular vesicles. Virions preattached to HeLa cells at $4^{o}C$ were not sensitive to treatment with anti-penton base serum. It is suggested that anti-penton base antibodies prevent the virions that enter the cells via vesicles from leaving these structures. Possibly only virions entering the cells via direct penetration could in this case manifest an infection. Virions attached to HeLa cells at low temperature were sensitive to treatment with antihexon serum thus indicating a neutralizing mechanism different from the ones described above. Kinetic analyses revealed that the antihexon neutralization curve was biphasic and no lag period was detected. Steady state antihexon neutralization values revealed that the experimental data fully fitted a theoretical curve based on a Poisson distribution of virus-antibody association, this curve indicates a single-hit model for antihexon neutralization.

INTRODUCTION

Measurement of antibody-mediated neutralization of viruses, i.e. the loss of virus infectivity, is a two-step procedure. In the first step, virus is mixed with antibodies which render the virus non-infectious, and in the second step the survival of virus is determined by employment of an appropriate cell-system and titration assay. This implies that three variables have to be considered, i.e. the virus, the antibodies and the host cells. As will be shown in the following, variation in each of these variables will markedly influence the outcome of a neutralizing reaction.

Kinetic curves

When analyzing the kinetics of a neutralizing reaction, virus is mixed with antibodies and incubated at an appropriate temperature. At regular times, usually one to five minutes, samples are withdrawn, diluted and thereafter analyzed for residual virus infectivity by e.g. plaque titration. In the reaction mixture, the antibodies are in such excess over the virus particles that the amount of antibodies consumed by attachment to virus is negligible, thus approximating the neutralizing reaction to a first order reaction (109). Inactivation of virus infectivity can be expressed by: $\frac{C_t}{C_0} = 1-(1-e^{\frac{-kt}{D}})^n$, C_0 and C_t are the concentrations of infectious virus at times zero and t, respectively; t is time in minutes, D is the final dilution of serum in the reaction mixture, k is the neutralization rate constant (min^{-1}) and n is the number of hits required for neutralization of one virion (66). In the case where one antibody inactivates one virus particle, i.e. n=1,

the equation is reduced to: $\frac{C_t}{C_0} = e^{\frac{-kt}{D}}$.

Plotting survival (C_t / C_0) on a logarithmic scale against time yields a straight line, as shown in fig. 1a (immediate decline), which however sometimes is preceded by a lag-period (delayed decline, fig. 1b).

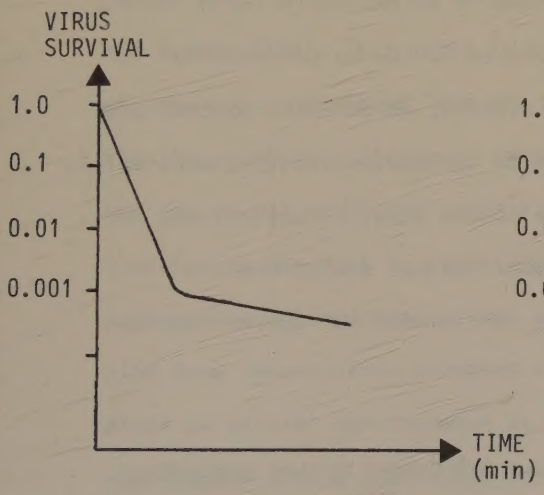

Fig. 1a: Single-hit curve

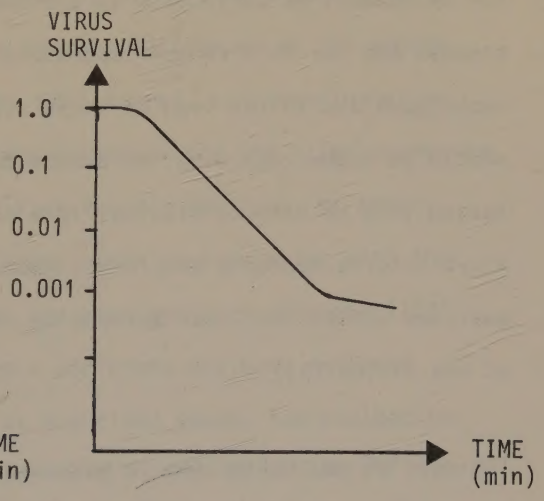

Fig. 1b: Multi-hit curve

Due to the immediate decline in fig. 1a, this curve is often interpreted as being the result of the combination of a single neutralizing antibody with each virus particle (single-hit mechanism) (16, 25, 60, 66, 97). The lag-period in fig. 1b indicates that more than one antibody is needed for neutralization of one virus particle and the length of this period is proportional to the number of antibodies needed (66). First order neutralization kinetics have been described for a variety of animal viruses such as poliovirus (16, 33, 60, 88), western equine encephalitis virus (WEE) (10, 16), Venezuelan equine encephalitis virus (VEE)

(28), Newcastle desease virus (NDV) (25, 97), influenza virus (51) as well as in bacterial viruses such as phages T2 and T5 (106) and f2 (15, 96). One may conclude that the most common type of survival curve is the single-hit curve, but in some cases multi-hit curves have been observed e.g. in the rabbit pox virus system (51). As pointed out by Daniels (12) and Della-Porta and Westaway (13), the attachment of antibodies to virions is an extremely rapid process and for this reason impossible to follow by conventional techniques and in the view of these authors, no definite conclusions should be drawn regarding the amount of antibodies required when solely basing this on kinetic studies. In a recent study of poliovirus, the kinetic curve showed a single-hit mechanism but stoichiometric analyses showed that four antibodies were needed for neutralization of one virion (33).

The rate of neutralization is markedly influenced by the concentration of serum. Lower concentrations of antibodies yield a correspondingly lower rate of neutralization (16, 25, 28, 60). Lafferty (51) observed that in the case of influenza virus an antiserum at a dilution of 1/20 yielded a single-hit curve,while a dilution of 1/40 showed a multi-hit curve, again demonstrating the rapidness of the initial combination between antibodies and virions in concentrated solutions.

When the temperature is lowered, the rate of neutralization is retarded (16, 28, 60, 88) and in some cases the temperature drop is accompanied by a shift from a single-hit to a multi-hit curve as demonstrated in the WEE system (10, 16).

It has also been shown that poliovirus followed first-order kinetics when neutralized by IgG antibodies, whereas IgM-neutralized poliovirus did not follow first order kinetics (88). Ozaki (78) showed that first order kinetics was only obtained with antisera collected more than 23 days after immunization of a rabbit with poliovirus. This observation parallels the shift in the production of antibodies from IgM to IgG as described by Svehag and Mandel (107) and Svehag (108). This shift is however influenced by the amount of immunogen inoculated into the host animal. Low dose immunization in a rabbit yields only IgM-antibodies and the titer increases for one to two weeks and thereafter rapidly declines. No memory function in the host is established since restimulation with the same amount of immunogen yields the same antibody-response (107). High dose immunization provokes a different antibody response. After an initial IgM-response as described above, IgG-antibodies soon appear with a continuous increase in both titer and affinity for some weeks. The increase in affinity is accompanied by a shift in the electrophoretic mobility of the antibodies from the γ_1 to the γ_2 -region (108). Restimulation with a high dose of immunogen yields an antibody response at an even higher rate and level as compared with the first stimulation, which shows that a memory function is established (107). One way to circumvent these difficulties could be to add an adjuvant to the immunogen before immunization, a procedure which preferentially yields high affinity antibodies (86).

Multiplicity curves

A constant amount of virions is mixed with different concentrations of antiserum and after incubation for several hours or days, the

surviving virus is determined. If log survival is plotted against the concentration of antibodies, a multiplicity curve is obtained (16). If a straight line is obtained, originating from the origin of the plot, it could be indicative of a single-hit mechanism.

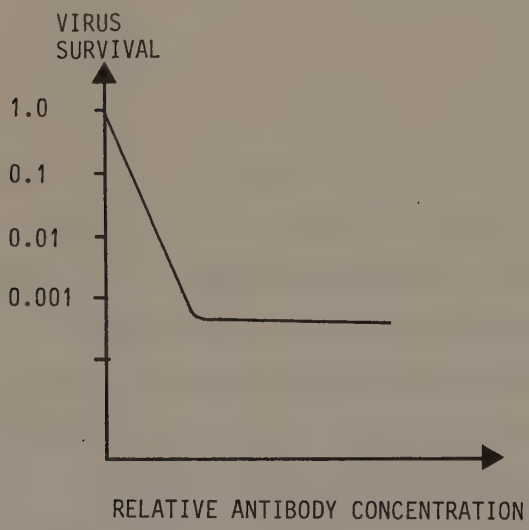

Fig 2: Multiplicity curve

Multiplicity curves as drawn in fig. 2 have been shown for VEE (29), NDV (25), WEE (16), and poliovirus (16,60).

The persistent fraction

So far it has only been dealt with the descending part of the kinetic curves. The exponential death rate usually levels off and the curve approaches a constant value. It must be emphasized that the concentration of antibodies is in great excess over the virus particles in most kinetic experiments, and yet a certain fraction of virus resists neutralization. This virus population has been called the persistent fraction (16) and is observed in both kinetic and multiplicity curves and has been reported for virtually all virus systems examined e.g. Japanese encephalitis virus (JEV) (35), vesicular stomatitis virus (VSV) (40, 113),

WEE (16), poliovirus (16, 60, 78, 88), VEE (28, 29), influenza virus (51), rabbit pox virus (51), vaccinia virus (57), NDV (25, 34, 97), reovirus (117) and herpesvirus (117). The mechanism behind the existence of a persistent fraction is not fully understood, although some hypotheses exist.

Addition of more antibodies to the surviving virus does not reduce the level of the persistent fraction (16, 25, 29, 54, 76) and addition of more virus to the persistent fraction yields an almost identical pattern of neutralization as initially obtained (16, 25, 34, 51, 76). Thus, a lack of antibody as the cause of the persistent fraction can be ruled out. The existence of a genetically stable subpopulation among the virus, which is resistent to the action of neutralizing antibodies has also been excluded (16, 25, 29, 34, 40, 57, 76, 113). No free infectious nucleic acid has been found in the persistent fraction (25, 29). A number of parameters that influence the level of the persistent fraction have been identified. Kjellén and Schlesinger (40) demonstrated that the outcome of a neutralizing reaction in the VSV system was markedly influenced by the cell system used for the determination of surviving virus. The same findings were made by Lafferty (51) in the rabbit pox virus system and by Lewenton-Kriss and Mandel (54) in the poliovirus system. The level of the persistent fraction is also affected by the experimental host employed for antibody production (51) as well as by the length of the period post immunization after which antisera are collected. "Young antisera", i.e. antisera obtained one to two weeks after immunization usually yield higher persistent fractions than antisera collected later on (54, 78). Wallis and Melnick (117) showed that filtration of the virus suspension prior to reaction with antibodies eliminated the persistent fraction

in a variety of virus systems and they propose that the persistent fraction emanates from viral aggregates, which in the center contain virions thus escaping the reaction with antibodies. Further studies on filtered virus have shown that the level of the persistent fraction is either somewhat reduced (29, 54) or is not affected at all (34, 57).

A most dramatic decrease in the level of the persistent fraction is obtained if anti-IgG-antibodies, i.e. antibodies directed against the antibodies used in the initial neutralizing reaction, are added to the persistent fraction (29, 34, 35, 54, 57, 76), thus indicating that the persistent fraction consists of infectious virus-antibody-complexes. Such virus is said to be sensitized as proposed by Notkins et al. (76).

Although some conflicting data exist, it is generally held for true that the attachment of antibodies to virus is an equilibrium reaction. However, under "normal" conditions, such as at physiological ionic strength and at pH-values around neutrality, the rate of dissociation is negligible. Thus, virus-antibody complexes are not dissociated upon dilution (10, 16, 25, 28, 60), although partial dissociation have been reported in some instances (35, 51, 97). Early antisera contain mainly low-affinity antibodies, which are more sensitive to dissociation upon dilution than are later collected antisera (12). One hypothesis for the occurrence of the persistent fraction argues that non-neutralizing antibodies within an antiserum bind to non-critical sites on the virus particle, and thereby sterically hinder subsequent neutralizing antibodies from reaching the critical sites. These critical

sites could however be covered by addition of anti-IgG-antibodies with a concomitant loss of virus infectivity (12, 29, 59, 76). The hypothesis described above may be exemplified by the influenza virus system. These viruses have two externally oriented glucoproteins; the hemagglutinin (HA) and the neuraminidase (NA) of which the former stimulates the production of neutralizing antibodies (118). Antibodies against the NA do not neutralize influenza virus but are able to sensitize, i.e. addition of anti-IgG-antibodies neutralizes influenza virus infectivity. One explanation for this phenomenon, as proposed by Majer and Link (58), is that anti-NA-antibodies attach to non-critical sites on the virus particle. By addition of anti-IgG-antibodies, these bind to the attached anti-NA-antibodies on the surface and thereby sterically cover the critical sites consisting of the hemagglutinin. The observation that sensitization precedes neutralization (i.e. addition of anti-IgG-antibodies immediately after mixing virions with neutralizing antibodies increases the rate of neutralization several times) is explained by the fact that the number of non-critical sites exceeds the number of critical ones (57, 59, 93). According to another hypothesis based on an observed heterogeneity in the antibody population, sensitization is an intrinsic step in the neutralizing reaction, but due to the low affinity of some antibodies the neutralizing reaction is not always completed and thus leaves a persistent fraction. Addition of anti-IgG-antibodies stabilizes the initially and often reversibly bound antibody (35, 59). The latter hypothesis is based on Lafferty's observation (52) that monovalent, neutralizing antibody fragments (Fab, see below) reversibly bind to influenza virus and dissociate upon dilution. The combination of virus and intact bivalent IgG-antibodies is

initially reversible upon dilution, but after prolonged incubation the union becomes irreversible. Lafferty suggests that this is due to an initially monovalent combination between virus and one of the antigen-binding sites of the antibody, and the shift to irreversibility is due to the combination of the other antigen-binding site, thus leading to bivalent attachment and stabilization of the antibody-virus linkage (52, 53). According to Lafferty (52) the activities of low affinity antibodies resemble in much the action of Fab-fragments.

Contradictory to both hypotheses mentioned above is the finding that monoclonal antibodies also yield persistent fractions (34, 113). In this case, the antibody population is homogenous regarding both affinity and binding specificity. Thus, non-neutralizing interfering antibodies are not available in these systems (113) and other explanatory mechanisms have to be found e.g. the existence of an equilibrium between attached and non-attached antibodies as proposed by Volk et al. (113). This hypothesis is supported by earlier findings that the addition of UV-inactivated virus to a neutralized virus-antibody mixture, partially restored the infectivity of the neutralized virus (16, 25).

Finally, it must be emphasized that the existence of a persistent fraction is not an artefact created in the assay system when mixing antibodies with viruses, since this phenomenon is also reported to exist in vivo. Gudnadóttir and Pálsson (26) demonstrated that visna virus coexists with neutralizing antibodies in the blood of infected sheep and Notkins et al. (76) reported that

lactic dehydrogenase virus in mice were covered with antibodies and were sensitive to inactivation by anti-IgG-antibodies.

Neutralization by antibody fragments

The combination between neutralizing antibodies and virions does not lead to an irreversible damage to the virus particle, since it is possible to recover full infectivity of neutralized virus by treatment of the virus-antibody mixture under conditions of low or high pH-values (25, 60). Papain digestion of antibodies splits the molecule into three pieces; two antigen binding fragments (Fab) and one fragment designated Fc, without antigen binding capacity. Papain digestion of neutralized poliovirus regains almost full virus infectivity, thus indicating the importance of a bivalent attachment of antibodies to the virus (18, 33, 39). Further, addition of anti-IgG-antibodies to the papain digested poliovirus-antibody mixture reneutralizes the viruses, thus indicating that the Fab-fragments still are attached to the virions (18, 39).

Pepsin digests a portion of the antibody molecule, yielding a bivalent fragment devoid of the Fc-region. This fragment is designated $F(ab')_2$, which upon sulfhydryl reduction may be split into two Fab'-fragments. In the WEE system (10) and in the poliovirus system (39) it was reported that the $F(ab')_2$ neutralized to the same extent as intact IgG-antibodies while the Fab'neutralized poorly. Again, neutralization could be restored by the addition of anti-IgG-antibodies (39). There seems to be an inversed correlation between the capacity to neutralize and the degree of degradation of the antibody molecule (24). Usually 90% or more of the neutralizing capacity is lost upon degradation into Fab- or Fab'-fragments. $F(ab')_2$-fragments usually

reveal an intermediate capacity to neutralize viruses and therefore these fragments occupy a position between intact IgG and Fab-fragments (15, 24, 96). An interesting observation was made by Stemke and Lennox (106) in their studies on the phage T2. Kinetic curves with intact IgG-antibodies showed a single-hit mechanism, while Fab-fragments revealed multi- hit-curves. Calculations performed by the authors showed that two to three hits were required for the neutralization of one phage in the latter case. However, a multi-hit curve was not obtained when phage T5 was analyzed in the same way (106).

Mechanisms of neutralization

As already pointed out, the attachment of antibodies to viruses does not irreversibly damage the particles, i.e. the inhibition of virus infectivity is likely to be found at some stages in the early interaction between viruses and the host cells such as adsorption, penetration or uncoating. One exception is virolysis, a complement-dependent neutralizing mechanism, which results in the breakdown of the envelope in some virus systems, with a concomitant loss of internal components.

One obvious way to neutralize virions is by aggregation, which is possible since antibodies are bivalent and virions multivalent. Aggregation is not always considered as being a "true" neutralization (65, 66). Recent studies with neutralizing monoclonal antibodies in the murine leukemia virus system (77) and in the mouse mammary tumor virus system (68) have shown that interference of antibodies with the attachment of viruses to the host cells is the most likely explanation for neutralization. But in many other

virus systems, attachment of neutralized virus to cells have been reported e.g. NDV (25, 104), rabbit pox virus (36), poliovirus (17,61), vaccinia virus (11) and influenza virus (90, 91). Inhibition of the final uncoating is reported for vaccinia virus (11) and for rabbit pox virus (36). In the latter virus system, penetration of neutralized virus into cells was markedly retarded. When studied in an electron microscope, neutralized NDV appeared to be degraded within intra-cellular vesicles (104).

Poliovirus is oscillating between two conformational states, A and B with isoelectric points of 7 and 4.5, respectively (63). Neutralizing antibodies stabilize the virus particles in the B configuration in which the particles are non-infectious. This appears to be an all-or-none phenomenon, since no particles are found at intermediate pI-values. There is a direct correlation between the degree of neutralization and increase in the proportion of B-particles and it has been stated that this is a single-hit phenomenon (64). The B-particles were found to be resistent to an in vitro uncoating system which was not the case with infectious A-particles (64, 67). Working with monoclonal antibodies against VP1, Emini et al. (18) confirmed that neutralization of poliovirus is accompanied by a decrease in pI. Treatment of virion-antibody complexes with papain regained full infectivity and the pI was raised to 7. By addition of anti-IgG-antibodies to the papaindigested virus-antibody mixture, virus was reneutralized and again the pI was lowered to 4.5. This emphasizes the role of bivalency for maintenance of the neutralized state. Neutralized poliovirus attaches and penetrates HeLa-cells (17, 61, 62) but no intact free virion RNA is found within the cells, and therefore uncoating seems to be blocked. Possee et al. (90, 91)

found that neutralized influenza virus attaches to, penetrates and uncoats in chicken embryo fibroblasts with kinetics being indistinguishable from that of normal virus. Uncoated virion-RNA is furthermore transported into the nucleus but herein transcription is blocked. It must be stressed that the envelope-associated hemagglutinin, with the attached antibodies, remains in the cytoplasm, but the inhibition of infection is found at the transcriptional level. The following explanatory hypothesis has been proposed: The hemagglutinin within the envelope is in physical contact with the transcriptase complex located in the interior of the virus particle. When antibodies attach to the hemagglutinins, a conformational change occurs, which is transmitted through the envelope to the transcriptase complex with a subsequent loss of activity (14, 91). Conformational changes after reaction with neutralizing antibodies have also been reported for bovine enterovirus (5) and indirectly for sindbis virus (9).

Single-hit contra multi-hit mechanisms

Neutralization of virus by a single antibody does not imply that only one antibody is attached to each virion; what it means is that only one is needed. The spokesmen for a single-hit mechanism have almost entirely suggested this mechanism based on kinetic curves (15, 25, 60). Recently Yoshino and Isono (123) proposed a model for neutralization of herpes virus, which is based on a single-hit mechanism. Attachment of one antibody leads to a series of physicochemical modifications in the virus particle, culminating in neutralization. A very interesting part in this theory is that it accounts for the heterogeneity among antibodies within an antiserum i.e. non-complement requiring antibodies can fullfil all the

modifications needed to achieve neutralization while complement requiring antibodies only succeed in initiating these modifications. This would explain both the occurrence of sensitized virus and of persistent fractions. Even though this hypothesis is based on neutralization of enveloped viruses, it could be extended to non-enveloped viruses in terms of low and high affinity antibodies.

The single-hit mechanism has been challenged by Daniels (12) and Della-Porta and Westaway (13). In the view of these authors the following facts are incompatible with a single-hit mechanism:

1. If the temperature of the reaction mixture is lowered or if the antiserum is diluted, kinetic curves may shift from single-hit to multi-hit. This shows that the reaction is very rapid and the amount of antibodies needed for neutralization of one virion can therefore not be measured by this technique.
2. The existence of a persistent fraction is incompatible with a single-hit mechanism.
3. Neutralization is increased by the addition of mediators such as anti-IgG-antibodies.
4. The outcome of the neutralization is in part dependent on the host cell system used for the determination of surviving virus.
5. The neutralizing capacity of two different neutralizing antibodies is greatly enhanced if the antibodies are mixed (synergistic effects). The same holds true for phenotypically mixed virus particles, which contain glucoproteins in their envelopes originating from both parental strains. The neutralizing capacity is enhanced when neutralizing antibodies directed to each parental strain is mixed and analyzed on the progeny virus.

6. At low concentrations of antibodies, some viruses such as flavivirus show an enhanced degree of infectivity. This enhancement is a step in the neutralizing reaction and takes place before enough antibodies have bound to achieve full neutralization.

Della-Porta and Westaway (13) therefore propose that many antibodies are needed for neutralization of one virion, and that the disposition of the bound antibodies over the viral surface is responsible for the cell-dependent outcome of a neutralizing reaction. The occurrence of a persistent fraction is due to steric barriers resulting from improper attachment of some antibodies and thereby too few antibodies are able to attach and therefore infectivity remains.

Synergistic effects have been reported in some virus systems. Monoclonal antibodies against a "neutralizing antigen" do not always neutralize virus infectivity, and sometimes a partial neutralization is obtained. If such partially neutralizing antibodies are mixed, enhancement in neutralizing capacity is obtained as reported for NDV (34), VSV (113), West Nile virus (79) and sindbis virus (7). In the latter two virus systems an enhancement in virus infectivity is also observed at subneutralizing concentrations of antibodies.

THE ADENOVIRUSES

The virions of the family Adenoviridae are non-enveloped DNA-viruses with a diameter of ca 80 nm. The family is subdivided into two genera; Mastadenovirus and Aviadenovirus due to the absence of any immunological cross-reactive proteins between the two groups (75). The genus Mastadenovirus contains adenovirus isolated from various species e.g. human, cattle, pig, sheep and dog. Today, 41 human adenovirus types have been isolated (Ad1-Ad41) (85, 116). They cause a variety of respiratory and eye infections (116). Human adenoviruses have been divided into subgroups based on their ability to agglutinate rhesus monkey or rat erythrocytes (95) or based on their oncogenicity in newborn hamsters (32). Highly or moderately oncogenic types are found in groups A and B, respectively. The non-oncogenic types (originally subgroup C) have been further divided into subgroups C and D based on immunological differences in their T-antigens (70). Further subdivisions have also been proposed (see ref. 116). Adenovirus type 2 is the type-virus for the genus Mastadenovirus (75) and is non-oncogenic but has the capacity to transform non-permissive cells (70).

The adenovirion consists of a nucleoprotein complex surrounded by an icosahedral capsid consisting of 252 capsomers. Of these, 240 are surrounded by six neighbor capsomers and are therefore called hexons (polypeptide II) (23, 111). The remaining capsomers are located in the twelve vertices and since they are surrounded by five neighbor capsomers, these structures are called the pentons. Each penton consists of a pentonbase (polypeptide III) and an antenna-like projection called the fiber (polypeptide IV) (23, 111). Two forms of hexons can be distinguished in the virus particle;

groups of nine-hexons, which form the triangular facets of the capsid and the peripentonal hexons, which surround the pentons. The hexon contains a group-specific determinant (α), common to almost all types within Mastadenovirus (31). In addition, the hexon has been reported to possess type-specific (ε), subgroup- and intersubgroup determinants (47, 72, 73, 84, 120).

The pentonbase has a group-specific determinant designated β besides subgroup and intersubgroup specific determinants (2, 72, 83, 111, 114). The fiber contains a type-specific determinant (γ) and also subgroup (δ) and intersubgroup specific determinants (43, 72, 82, 111, 114). Three proteins, protein VI, VIII and IX are associated with the hexons (19), but protein VI also seems to be associated with the internal core-structure (56, 99). In the vertex region, in close connection with the pentonbase, protein IIIa is situated (19, 21). The internal core-structure consists of double-stranded linear DNA, to which three basic proteins are attached. Two of these are proteins V and VII (19, 56) and the third is protein IVa_2 which is attached to the 5'-ends of the virion DNA (94). Most of the structural proteins are synthesized in great excess in the infected cells and could be isolated and separated by e.g. ion-exchange chromatography (50), together with a variety of non-structural proteins. Of the latter, the two most abundant are the 100K protein, a late non-structural protein which is involved in the assembly of hexons (6) and the 72K protein, a DNA-binding protein involved in DNA replication (55, 112).

Neutralization of adenovirus

The reaction between adenovirus and neutralizing antibodies does not differ markedly from other virus systems. The kinetic curve shows an exponential decline in surviving virus followed by a decrease in death rate (compare fig. 1). The initial combination between virus and antibodies is a very rapid process and is completed within minutes (42). It has been shown that the second part in the kinetic curve is not due to combinations of more antibodies to the viruses, but rather to reactions taking place within the initially formed virus-antibody complexes (42). Thus, as in other virus systems, adenovirus neutralization is a multistep process. The rate of the neutralization is dependent on the concentration of antibodies and if enough antibodies are present, no persistent fraction is observed (42, 117). The formed virus-antibody complexes are stable and no evidence for reversibility have been found (42).

Reactivation of virus infectivity is obtained by treatment of neutralized virus-antibody complexes at low pH-values. This reactivation is not concomitant with a dissociation of antibodies from the virus particles (44).

The role of various adenoviral antigens in eliciting neutralizing antibodies when immunized into different host animals, has been discussed intensively in the past. An antihexon-serum comprises the major neutralizing activity (1, 27, 37, 47, 69, 72, 84, 103, 115, 120, 121), although the neutralizing capacity varies among different serotypes (115). In one report (81) it was claimed that an antihexon-serum did not neutralize virus infectivity. In a following report from the same author (84), the existence of two

different populations of hexons isolated from infected cells was reported. The neutralizing activity was preferentially confined to one of these hexon-populations (84, 115).

The role of the fiber antigen as an inducer of neutralizing antibodies is a matter of controversy. Thus, some investigators claim that an antifiber-serum indeed possesses neutralizing antibodies (1,37, 69, 120), while others report the corresponding lack of such neutralizing antibodies (27, 47, 115). In one investigation it was reported that an antifiber-serum neutralized virus infectivity as measured by the FFU-technique but not when assayed by the plaque-reduction technique (82). Usually the final reading in the FFU-assay is made after 48 hours, whereas the corresponding readings in the plaque-titration assay are performed at around two weeks. The results obtained with the FFU-titration therefore may include a delayed start of the multiplication process, due to interference of antibodies with e.g. penetration or uncoating. Later it was shown that the results obtained after prolonged incubation of antifiber-treated virions in the FFU-assay did not differ from the results obtained with the plaque-titration (89).

An antifiber-serum effectively aggregates virions at the zone of equivalence (21, 92, 111), and this fact always has to be considered when judging the neutralizing capacity of an antifiber-serum. Usually small amounts of virus are mixed with an excess of antibodies, under which circumstances no or minor aggregation occur (105) - a subsequent lack of neutralization is therefore noticed. If the relative concentration of virus is increased, a marked aggregation and subsequent neutralization is observed. These factors may in part

explain the contradictory opinions about fiber acting as a "neutralizing agent". However, studies in vivo have shown that administration of purified hexon or fiber into mice, protected the animals against a challenge lethal dose of adenovirus type 5 (69). Similarily in a study of humans, it was shown that immunization with purified fiber antigen stimulated the production of neutralizing antibodies, as also was the case after immunization with purified hexon (1). In another example occular challenge with homologous virus showed that the antifiber-immunized volunteers were almost totally protected, while a corresponding challenge in hexon-immunized volunteers showed a significant reduction in illness as compared with unimmunized control persons (37). Thus, even though many investigators classify fiber as being a "non-neutralizing antigen" when studied in vitro, the above mentioned experiments in vivo clearily show the protective role of the fiber as an immunogen.

Antipenton-sera have been shown to possess none or poor neutralizing capacity (27, 47, 83, 103, 115). In one study it was found that a moderately neutralizing antipenton-serum absorbed with purified fiber, no longer displayed any neutralizing activity, whereas an antifiber-serum displayed low neutralizing activity (72, 74). However, both antifiber- and antipenton base-antibodies were able to sensitize virions and neutralized infectivity independent of one another upon the addition of anti-IgG-antibodies (74). Thus, it is evident that each type of antibody is able to combine with virions without causing neutralization. When acting together, a synergistic effect is obtained which results in neutralization. A similar synergistic effect was reported by Hierholzer and Dowdle (30). An antihexon- and an anti-dodecon-serum neutralized separately

with low titer. When mixed, enhancement in neutralizing capacity by a factor of 8-16 was observed. The authors conclude that "maximal neutralization titers may involve antigen-antibody reactions at more than one site". Under ordinary conditions, neutralization of adenovirus is a serotype-specific phenomenon, i.e. different serotypes are neutralized only by homologous antisera (27, 30, 47, 69, 72, 120). However, minor neutralizing cross-reactions between different serotypes and total virion-sera have been reported (119). This cross-reaction appears to exist within, but not between subgroups (38). It is possible to separate the type-specific antibodies in a serum from the cross-reactive ones by means of passing the serum through a heterologous immunosorbent where any cross-reacting antibodies are retarded, while type-specific antibodies pass through. All neutralizing activity in an antihexon-serum, separated into cross-reactive and type-specific antibodies as described above, is found in the latter fraction (27, 121), and no neutralizing activity is detected among the cross-reactive antibodies (27, 121). Still these latter ones seem to play a role in adenovirus neutralization, since addition of heterologous antisera to a homologous virus-antibody mixture enhances the extent of neutralization, provided that the heterologous and homologous antisera emanate from virions within the same subgroup (47). This finding suggests the existence of subgroup-specific determinants in addition to the type- and group-specific ones. Moreover, heterologous antisera neutralize virions within the same subgroup provided that the reaction is allowed to take place at low pH-values, and heterologous neutralization is achieved at normal pH if anti-IgG-antibodies are added (46, 47). The authors suggest that either type-specific antibodies or low pH-values expose hidden subgroup-

specific determinants in the virion which allow cross-reactive antibodies to reach common sites and thus enhance neutralization. This hypothesis does not explain why heterologous antisera neutralize virions upon addition of anti-IgG-antibodies, but available data does not exclude the possibility of the existence of additional subgroup-specific determinants exposed at the outside of the virion. These sites are not targets for neutralization per se, but targets for added non-neutralizing heterologous antibodies which subsequently act as new targets for added anti-IgG-antibodies with a resultant neutralization. The added anti-IgG-antibodies could neutralize by a different mechanism e.g. by cross-linking the virus particle and thereby preventing an effective uncoating.

As mentioned above, two classes of hexons were isolated from infected cells, one of which eliciting the production of neutralizing antibodies (84, 115). The other class of hexons elicited antibodies which were able to sensitize virions and thereby neutralize virions upon addition of anti-IgG-antibodies (115). Shortridge (103) managed to isolate three different populations of hexons from urea-treated virions. Only two of these, the peripentonal and the adjacent hexons, elicited neutralizing antibodies when immunized into rabbits, although all three antisera displayed the same complement-fixing titer. Since neutralization is a type-specific phenomenon, this rises the question whether some classes of hexons in the virus particles are enriched in type-specific determinants active in the neutralization. Willcox and Mautner (122) did not find any differences between groups of nine- hexons, peripentonal hexons and hexons isolated from infected cells in their capability to bind cross-reacting and

type-specific antihexon-antibodies. The relative proportion of type- and cross-reactive sites on the hexons were the same in all three preparations. Thus all three preparations of hexons appear to be antigenically uniform. Recently it was proposed that the difference between the two classes of hexons isolated from infected cells, was due to proteolytic degradation of one of the classes (3) and since this class of hexons is absent from virions, a biological relevance of this hexon population has to be determined.

Since only a minor fraction of the antibodies within an antihexon-serum is type-specific (121), it has been speculated that the neutralizing reaction is due to binding of type-specific antibodies to critical sites on the virion which drastically alter the conformation of the capsomers and thus damage the virion (122).

Neutralization by antibody fragments

Papain-derived Fab- and pepsin-derived $F(ab')_2$-fragments obtained from either antihexon or antifiber-serum displayed no neutralizing activity in the adenovirus system (115). However, all fragments sensitize virions which demonstrates their ability to combine with virus particles (115). Fab-fragments from a total anti-virion serum was similarily shown to be devoid of neutralizing activity although the fragments were active in hemagglutination inhibition tests, thus revealing the capability of the fragments to combine with virions (43).

$F(ab')_2$-fragments from a total anti-virion serum were able to neutralize virus infectivity, although at a much lesser extent

than intact IgG (45). As mentioned above, neutralization of adenovirus with intact IgG-antibodies is a multistep procedure consisting of a) the attachment of antibodies to virions and b) secondary reactions within the formed virus-antibody complexes. Neutralization with $F(ab')_2$-fragments reveals a similar curve of neutralization kinetics as intact IgG during the first minutes, but thereafter a steady state is reached and apparently no secondary neutralization occurs. This could be due to the absence of the Fc portion from the $F(ab')_2$ - fragment (45).

THE PRESENT INVESTIGATION

This section is dealing with the following topics: the specificity of a neutralizing antiserum (I), the assessment of the extent of virus neutralization (II) and the neutralization of adenovirus (III, IV).

Specificity of a neutralizing antiserum (I)

In order to investigate the specificity of an antiserum obtained after immunization of highly purified adenovirus type 2 into rabbits, rocket immunoelectrophoresis was used to screen a DEAE-cellulose-chromatogram of soluble antigens remaining after virus isolation by preparative ultracentrifugation. This DEAE-separation procedure is established when it comes to the separation of the major soluble antigens from virus infected cells (50, 81). It was shown that a majority of the proteins found in virions were synthesized in excess and could be separated by DEAE-chromatography and antibodies against the following structural proteins were found in the total anti-virion serum: protein IVa_2, fiber, penton, proteins IIIa and V, penton base and hexon. Protein IVa_2 was identified in the volume of unretained material together with protein IX (not shown). Protein IIIa was coprecipitated with protein V and a connection between these two proteins in the virion has been described earlier (21). Thus, the only structural proteins not found in the chromatogram were proteins VI, VII and VIII. At least proteins VI and VIII have previously been shown not to be produced in excess (20). Besides the structural proteins, the antisera revealed the presence of two other immunoreactive proteins, the 72K and the 100K proteins. The latter was less surprising since this protein has

an established strong affinity for the hexon (98) and is often observed as a contaminant of virus particles when analyzed in SDS-polyacrylamide gels. The occurrence of antibodies against the 72K protein was however unexpected since this species has not been reported to exist within the virions and further, the rabbits used for antibody production are normally regarded as non-permissive for human adenovirus type 2. For the final identification of the protein an anti-72K-serum was produced and the specificity was compared with a reference-serum against the DNA-binding protein (DBP) (55) and the two sera displayed immunological identity against the DEAE-derived 72K-protein. A variety of immunological methods were used in attempts to detect the DBP within virions, and in no case any evidence for the existence of a virion-associated DBP was found.

To investigate why the DBP was produced in the rabbit upon immunization with purified virions, immunization with two differently disrupted virion-preparations were performed. The virions were disrupted either by 5M of urea or by repeated freeze-thawings (92). In neither case the disrupted virion-sera revealed antibodies against the DBP as judged from immunoblotting tests. It was therefore concluded that intact virus undergoes at least a partial replication in the rabbit, which includes the synthesis of the early DBP. This protein then acts as an immunogen in the rabbit thus yielding anti-DBP-antibodies.

Pereira and Kelly (80) reported the persistence of Ad5 in rabbits but could not repeat the same findings with Ad2, and Schrader and Wigand (100) showed that Ad5 but not Ad11 was able to multiply in

rabbit kidney cells. These important findings thus show that the ability to multiply is serotype-specific in different celltypes and always has to be considered when intact virions are used as immunogen in host systems normally regarded as non-permissive.

Assessment of the extent of virus neutralization (II)

The most frequently used assays for determining virus neutralization is FFU-titration (87), plaque-titration (41) and inhibition of cytopathic effect. The two latter methods, although very sensitive, both have the disadvantage of being very time-consuming; final readings are usually made after some weeks and thus very "healthy" cell cultures must be used. A possible drawback may be that any remaining neutralizing antibodies in the virus sample may greatly influence the outcome of the titration. The sensitive FFU-assay measures the production of virion-specific antigens as well as virions in infected cells and is less time-consuming since the final readings are usually performed after around 48 hours. In all three methods, monolayer cultures of cells are used and by washing the cells after adsorption of virions, cells may be released and virions not properly attached may be lost. Since neutralization could be defined as "inhibition of expression of the viral genome as indicated by the lack of formation of viral progeny" (115) we developed an immunological method which quantifies the total production of progeny virus from infected cells in suspension cultures.

This method we called PVIT i.e. progeny virus immunotitration. A mini-spinner culture is grown for each sample to be analyzed and virus neutralized by various serum dilutions is added at a multiplicity of infection of one infectious unit per cell i.e. the

minimal amount of physical particles needed for a successful infection of one cell. The virus-cell mixtures are then incubated for 39 hours, allowing infected cells to produce progeny virus. The infected cells are thereafter sonically treated and the total progeny virus produced in the spinner culture is isolated by a one-step CsCl-gradient centrifugation procedure. To the virus-material is added urea of a final concentration of 5M and disrupted virions are quantified by rocket-immunoelectrophoresis based on the total hexon content. By measuring the rocket heights, the corresponding values for amounts of virions are obtained from standard curves. The degree of neutralization is expressed as percent reduction in total virus yield in the sample as compared with control infections. The variation between different determinations of an identical partially neutralized sample is between 5-10% i.e. somewhat better than the 20% precision as reported for the plaque-titration assay.

Neutralization of adenovirus (III, IV)

For the purpose of these investigations, monospecific antihexon-, anti-fiber- and anti-penton base-sera were produced. Since it was reported by Norrby and Wadell (74) that antigenic sites exist on the virion which are absent among the corresponding soluble antigens used for immunization, a total anti-virion-serum absorbed with purified fiber or hexon or both hexon and fiber was included. To be able to compare the neutralizing titers of the two series, i.e. the monospecific and the absorbed polyspecific antisera, and since the titers usually are higher in monospecific antisera, the latter were diluted to contain the same amount of precipitating antibodies against the relevant antigen as compared with a total anti-virion-serum. Such titrations

were performed by rocket immunoelectrophoresis.

In the neutralization tests performed by the PVIT method, it was demonstrated that the total anti-virion-serum neutralized virus infectivity to the same extent as the monospecific antifiber-serum. When the antifiber-antibodies were removed from the anti-virion-serum, the remaining neutralizing capacity was the same as the antihexon-serum and when the anti-virion-serum was depleted of both antihexon- and antifiber-antibodies, the remaining neutralizing activity equalled that of the anti-penton base-serum. Thus, all three kinds of monospecific antisera displayed neutralizing activity. It was also shown that neutralization was not due to the formation of cytotoxic virus-antibody-complexes with subsequent killing of the cells (48, 49). Since no significant DNase-sensitivity among neutralized virus was detected, no direct antibody-mediated destabilization of such virus was anticipated.

The antifiber-serum effectively aggregated virions, which nevertheless were able to attach to HeLa-cells to an even greater extent than untreated control virus. It was previously shown that the initial uncoating of virus (here called primary destabilization) takes place already at the cell surface (110). Antifiber-neutralized virions were not as sensitive to primary destabilization as control virus, probably because virions within the aggregates did not reach the cell surface and thereby avoided the cell-mediated destabilization process. When analyzed in an electron microscope, it was demonstrated that among the attached antifiber-neutralized virions, 85% were found at the cell surface, whereas the remaining 15% were located as large aggregates within vesicles - probably lysosomes.

Virions attached to HeLa-cells at 4°C were not neutralizable by an antifiber-serum, thus supporting the proposal that antifiber-antibodies neutralize virions by mere aggregation.

In the case of anti-penton base-neutralization, a maximal value for neutralization was shown to be around 50%, even if antibodies were added in great excess. Anti-penton base-neutralized virions attached to cells and became destabilized to the same extent as untreated control virus. However, such neutralized virions were subsequently trapped within intracellular vesicles.

It appears as if adenovirus may penetrate the cell membrane by two alternative mechanisms. One is by direct penetration (4, 71) and the other is via an endocytotic mechanism (8, 22). It has further been shown that the penton base plays a crucial role for the ability of virions to leave an endosome (101, 102). It therefore seems plausible that anti-penton base-antibodies stoichiometrically cover the penton bases and thereby prevent such virions from leaving these vesicles. Only virions entering via direct penetration could in this case manifest an infection. A possible multi-hit mechanism for anti-penton base-neutralization fits well with the above mentioned observations and with the fact that virions already attached to HeLa-cells were not sensitive to treatment with anti-penton base-antibodies.

Antihexon-neutralized virions appeared to attach to cells in a normal way. The cell-mediated destabilization of neutralized virions on the cell surface was indistinguishable from that of untreated control virus. However, following penetration neutralized virions were confined to intracellular vesicles. Virions attached to HeLa-cells were to a great extent neutralizable by an antihexon-serum. It therefore

seems as if the antihexon-mediated neutralization acts by a mechanism, different from the ones mentioned above, i.e. aggregation and stoichiometric covering of viral structures. Kinetic analyses of antihexon-neutralization revealed no lag period, not even if the antiserum was highly diluted or if the temperature was lowered to 4°C. These observations are all indicative of a single-hit mechanism. The same conclusion could be drawn from multiplicity curves, based on steady state neutralization values. A straight line was obtained, originating from the origin of the plot and Poisson calculations showed that the experimental neutralization data were equivalent to single-hit values. However, the possibility must be kept in mind, that several different species of antihexon-antibodies could be involved in the neutralization process and if one species is present in limiting concentrations, this could lead to false single-hit values.

The kinetic curve was biphasic, starting with an exponential decline in viral survival, followed by a phase with a decreased rate of neutralization. The first part of the kinetic curve could be the result of a successful combination between one or few antibodies with one virion, thus resulting in a rapid loss of virus infectivity. The other part of the kinetic curve could be a consequence of an improper combination between the reactants resulting in infectious virus-antibody complexes, which subsequently become neutralized as a possible result of rearrangements of the antibodies within these complexes. Infectious virus-antibody complexes could also be neutralized by addition of anti-IgG-antibodies; virion aggregation is most likely the mechanism behind this neutralization. Non-neutralizing antihexon-antibodies coexist with neutralizing antibodies within an antihexon-serum.

Complexes between virions and the former antibodies are also neutralizable upon addition of anti-IgG-antibodies.

If only one or few antibodies are involved in antihexon-mediated neutralization of one virion, it is tempting to speculate about a conformational change of the viral capsid as a part of the neutralizing mechanism. Such a conformational change could e.g. mask the penton bases with a concomitant loss of infectivity following the same mechanism as described above for anti-penton base-neutralization. Conformational changes as a part of a neutralizing mechanism have great support in the literature and has been described in a variety of virus systems.

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I.

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Human adenovirus 2 as immunogen in rabbits yields antisera with high titers of antibodies against the nonstructural 72K DNA-binding protein

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Summary

Immunization in rabbits with intact, highly purified adenovirus type 2 (Ad2) virions, yielded antisera with high titers of antibodies against the 72000 dalton DNA-binding protein (DBP). This was established by rocket immunoelectrophoresis when an anti-intact Ad2-antiserum was analyzed against fractions from an ion-exchange chromatogram of soluble antigens remaining after virus isolation from virus-infected HeLa cells. The high anti-DBP titer did not reflect the composition of the immunogen, since no DBP was detectable within virions. An antiserum raised in response to mildly disrupted virions showed no specificity against the DBP, but contained antibodies against the same structural proteins as the anti-intact Ad2-antiserum, when analyzed by the immunoblotting technique. These findings indicate that the nonpermissive rabbit as an experimental host permits early gene expression of a human adenovirus.

adenovirus 2, DNA-binding protein, soluble antigen, rabbit immunization, immunoblotting, early gene expression

In the adenovirus system large amounts of virus specified proteins are synthesized in excess in the productively infected cell, and these proteins are separable under non-denaturing conditions by ion-exchange chromatography. In this way both structural and nonstructural proteins have been isolated and characterized (Petters-

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son and Wadell, 1985). One of the latter is the phosphorylated, 72000 dalton single-stranded DNA-binding protein (DBP) (Linné et al., 1977). DBP is a multifunctional protein since it has been reported to be involved in DNA replication and in both early and late gene expression (Flint, 1980).

HeLa cells were maintained in suspension cultures as recently described (Persson et al., 1983). Approx. 4×10^8 cells were synchronously infected with Ad2 at a multiplicity of 500 particles per cell according to Everitt et al. (1971), and at 14 h post infection (p.i.) [^{35}S]methionine (2.5 μCi/ml; Amersham International Ltd.) was added. At 40 h p.i. virus-infected cells were sedimented and virus was purified by a procedure including two CsCl-gradient centrifugations and one rate separation in sucrose gradients ranging from 10 to 25% (w/v) and with the addition of 1% Triton X-100 to the light sucrose solution at the stage of gradient mixing (modified from Sundquist et al., 1973). Such three-step purified virus was used for immunizations in rabbits (unlabeled virus) or as markers in electrophoretic analyses ([^{35}S]methionine-labeled virus).

To evaluate the degree of possible host cell protein contamination in the virus preparations used for immunizations the following experiment was carried out: uninfected cells were separately labeled for 2.3 to 3 generations with either [^{3}H]thymidine (0.2 μCi/ml) or a [^{3}H]amino acid mixture (2.5 μCi/ml; both isotopes were from New England Nuclear) and mixed with equal amounts of unlabeled Ad2-infected cells. The cell mixtures were subjected to virus purification as described above, and Table 1 shows the cellular contamination measured as trichloroacetic acid-precipitable radioactivity at the different stages of purification. The maximum level of cellular protein contamination was thus about 4 ng per OD unit of virus, assuming that 1 OD unit equals 0.28 mg protein (Maizel et al., 1968).

Antisera against the intact three-step purified Ad2 were obtained after three successive intramuscular immunizations in rabbits over a four week period (Harboe and Ingild, 1973), with 1.4×10^{12} virions each time, corresponding to 0.39 mg

TABLE 1

CELLULAR CONTAMINATION MONITORED DURING Ad2 PURIFICATION WHEN UNLABELED Ad2 INFECTED CELLS WERE MIXED WITH EITHER [^{3}H]THYMIDINE OR [^{3}H]AMINO ACID-LABELED UNINFECTED CELLS

Purification step	Total cpm ([^{3}H]thymidine)	Total cpm ([^{3}H]amino acids)
Starting material	2.88×10^7 (100) [a]	1.29×10^8 (100)
Supernatant after 10000 $\times$ *g* centrifugation	1.67×10^7 (58)	6.86×10^7 (53)
Double CsCl cushion	4.13×10^4 (0.14)	1.82×10^5 (0.14)
Sucrose–Triton X-100 gradient	997 (0.0035)	1.73×10^4 (0.013)
CsCl gradient	52 (0.00018)	1.86×10^3 (0.0014)

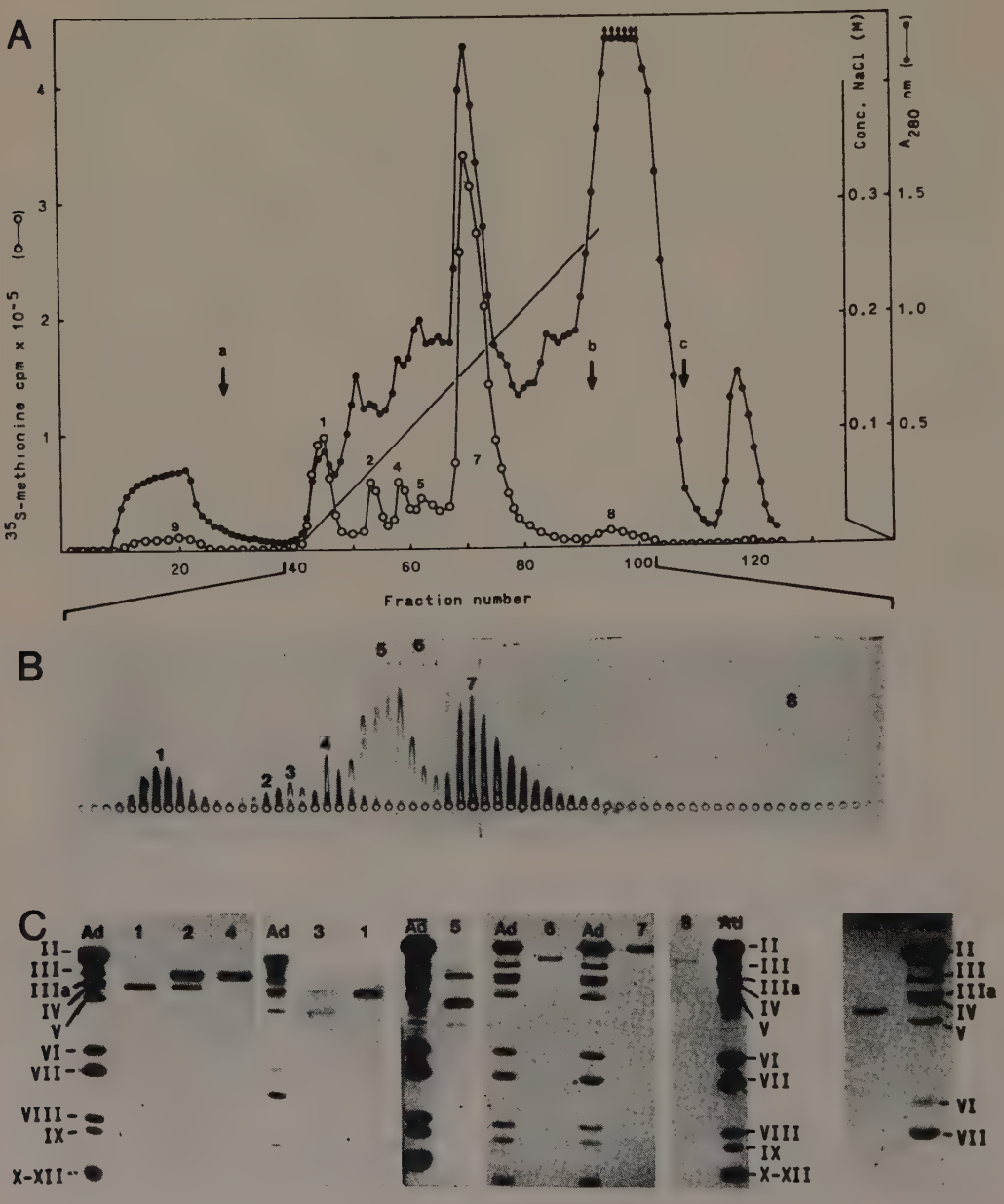
[a] The figures within parentheses refer to the percentage of host cell contamination.

protein. The immunogen was added an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) at the first and second immunization. To obtain an antiserum against disrupted virions, 1.4×10^{12} virions from the same virus pool were dialyzed against 25 vols. of 5 M urea for 1.5 h at room temperature. After removal of the urea by dialysis against phosphate-buffered saline (PBS), rabbits were immunized according to the schedule above. Treatment of naked viruses with the proteotrophic agent urea is an established method to inactivate virions and still retain most of the immunogenicity of the proteins.

The anti-intact Ad2-antisera were analyzed by rocket immunoelectrophoresis (Laurell, 1966) against the virus specified proteins synthesized in excess in infected cells. For this purpose ^{35}S-labeled soluble antigens, recovered after virus purifications, were dialyzed against 50 mM Tris-HCl buffer, pH 7.75 and subjected to separation by ion-exchange chromatography on DEAE-cellulose (DE 52, Whatman Inc., Clifton, NJ, U.S.A.) essentially as described by Pettersson et al. (1967), and a typical elution profile is shown in Fig. 1A. Indicated fractions in Fig. 1A were analyzed by rocket immunoelectrophoresis against an anti-intact Ad2-antiserum (Fig. 1B). [^{35}S]Methionine-labeled protein material from the indicated and numbered fractions in Fig. 1B was separately immunoelectrophoresed and the uppermost parts of 15–30 identical rockets were cut out; antigen–antibody complexes were eluted in 150–250 μl of SDS-disruption solution (Maizel, 1971) at 60°C for 2 h, whereafter the extracts were boiled for 3 min and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1C). In order of increasing salt concentration in the chromatogram the fiber antigen (peak 1) eluted first, followed by the penton (peak 2) and the penton base (peak 4). Protein IIIa eluted as a distinct peak (No. 3) overlapping the penton. SDS–PAGE analysis of this material revealed a coprecipitation of polypeptide IIIa and core polypeptide V, which also was confirmed by double immunodiffusion against monospecific anti-IIIa and anti-V antisera (not shown). However, the bulk of protein IIIa was pelleted at the $10\,000 \times g$ centrifugation step during virus purification (not shown). Between the penton base and the major peak of radioactivity, comprising the hexon antigen (peak 7), two species of immunoreactive proteins were detected (peaks 5 and 6). The immunoreactive protein (peak 6), both preceding and overlapping the hexon antigen and further appearing as a small peak (No. 8) within the major UV-absorbing nucleic acid peak, was identified as a polypeptide in the 100,000 dalton molecular weight region. Most likely this protein is the previously identified 100K protein since one of its elution positions in the chromatogram equals that obtained by Oosterom-Dragon and Ginsberg (1980). The other precipitate (peak 5) eluted at a position corresponding to 0.12 M NaCl and revealed upon SDS–PAGE analysis three polypeptides with molecular weights of around 72 000, 43 000 and 30 000 (Fig. 1C). The relative elution position in the chromatogram of this material, together with the pattern of the labeled polypeptide content in SDS–PAGE, suggested that this protein corresponded to the non-structural 72K DBP (Rosenwirth et al., 1976; Linné et al., 1977).

To test this hypothesis, rocket immunoprecipitates developed between the protein material in peak No. 5 in Fig. 1 and the anti-intact Ad2-antiserum were cut out.

These were formed in a low gelling temperature agarose (Sea Plaque Agarose, FMC Corporation, Marine Colloids Division, Rockland, ME, U.S.A.). The gel pieces were melted at 65°C for 15 min and the precipitates were sedimented and washed with PBS. The precipitates were suspended in 0.5 ml of PBS and added 0.5 ml of Freund's complete adjuvant, and then injected into a rabbit as described above.



Thirty immunoprecipitates yielded 40 μg of total protein per immunization. This serum was analyzed and compared with an established reference anti-Ad2 DBP-antiserum (Linné et al., 1977). These two antisera, together with an anti-intact Ad2 antiserum, showed complete immunological identity in double immunodiffusion against a preparation of the 72K protein derived from a DEAE-cellulose chromatogram (Fig. 2). In the following we therefore refer to the peak No. 5 material as the 72K protein.

Immunoblottings were performed essentially as described by the manufacturer of the Zeta-Probe blotting membranes (Bio-Rad, Chemical Div., Richmond, CA, U.S.A.), and the double antibody technique was employed to study the specificities of the anti-intact Ad2 and anti-urea-dialyzed Ad2 antisera. As shown, both antisera possessed the same specificity regarding all structural proteins of the virions (Fig. 3). A notable feature of the immunoblotting assays was the fact that polypeptides of the basic core proteins, i.e. V and VII, had a tendency to detach from the Zeta-Probe membranes. The major difference between the two sera is demonstrated in lane 2, where the applied material was derived from peak position No. 5 in a DEAE-chromatogram (Fig. 1) and containing unlabeled 72K protein together with traces of penton antigen. The anti-intact Ad2 antiserum contained antibodies against the 72K protein and marked this polypeptide heavily (Fig. 3B), whereas the anti-urea-dialyzed Ad2 antiserum was totally devoid of antibodies against this polypeptide species (Fig. 3A). The same result was obtained with an antiserum against Ad2 virions inactivated by repeated cycles of freeze-thawings (Prage et al., 1970), in which no antibodies against the 72K protein could be detected (not shown). A number of immunological methods turned out negative in assays for direct confirmation of the DBP within disrupted virions. The methods include the immunoblotting technique, crossed immunoelectrophoresis and *Staphylococcus aureus* mediated im-

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Fig. 1. (A) A DEAE-cellulose chromatogram of soluble antigens from Ad2-infected cells. [^{35}S]Methionine-labeled antigens remaining after virus isolation were separated on DEAE-cellulose in 50 mM Tris-HCl, pH 7.75. A NaCl gradient (0–0.4 M) was applied at arrow a. At arrows b and c, 0.5 and 2 M NaCl was applied, respectively. In assays for radioactivity, 20 μl samples were precipitated on paper filter discs with 10% trichloroacetic acid and counted in a scintillation cocktail based on toluene–Omnifluor (0.4%; New England Nuclear). (B) Rocket immunoelectrophoresis of indicated fractions in A. The agarose gel contained 5% of an anti-intact Ad2-antiserum obtained in response to three-step purified virions. The gel consisted of 1% agarose (Type 1, Sigma Chemical Co.) in 73 mM Tris/24 mM barbitone buffer, pH 8.6, and 0.45 mM calcium lactate. (C) The numbered rockets in B were cut out and solubilized as described in the text. SDS–PAGE analyses were performed on slab gels (1.5 × 70 × 70 mm) with 13% acrylamide and 0.35% bisacrylamide essentially as described by Maizel (1971). Gels were processed for fluorography (Bonner and Laskey, 1974) and after drying, the gels were exposed to Kodak XG/14 X-ray films for 3–30 days at −70°C. The indicated gel lanes are numbered in accordance with the rocket immunoprecipitates of B. The lanes contain the following polypeptides, according to the nomenclature of Anderson et al. (1973): 1, IV (fiber); 2, III and IV (penton); 3, IIIa and V; 4, III (penton base); 5, the DBP, i.e. 72K, 43K and 30K polypeptides; 6 and 8, the 100K protein; 7, II (hexon). Lane 9 shows an immunoprecipitate from the material not retained by the ion-exchanger shown in A. The polypeptide corresponds presumably to polypeptide IVa_2, which is the protein linked to the ends of adenovirus DNA (Rekosh et al., 1977). Ad = an [^{35}S]methionine-labeled adenovirus type 2 polypeptide marker. For the polypeptides of the hexon, penton base, IIIa, V and VII the following molecular weights were used: 120000, 85000, 66000, 48500 and 18500, respectively (Anderson et al., 1973).

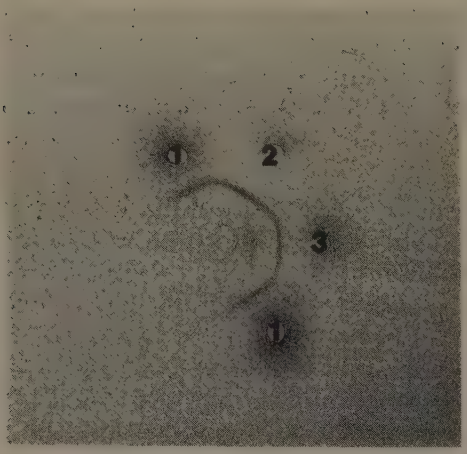

Fig. 2. Double immunodiffusion between the 72K protein and different antisera. The diffusion was performed in 0.8% agarose in PBS. The wells contain the following reagents: 1, 15 μl anti-72K antiserum; 2, 7.5 μl reference anti-Ad2 DBP antiserum; 3, 15 μl anti-intact Ad2 antiserum. The center well contains 15 μl of the 72K protein peak from a DEAE-cellulose chromatogram (peak No. 5 in Fig. 1A). The gel was stained in 0.2% Coomassie brilliant blue R (CBB-R).

munoprecipitation of disrupted virions with anti-72K antibodies. The results obtained with the antiserum against freeze-thawed virions therefore exclude the possibility that the absence of anti-DBP antibodies from the anti-urea-dialyzed Ad2 antiserum was due to specific urea denaturation of nondetectable amounts of DBP within the virus particles.

Based on these findings we suggest that human adenovirus 2 undergoes a 'partial replication' in the rabbit, which at least implies the activation of early gene region 2 encoding the established early nonstructural 72000 dalton DNA-binding protein, which subsequently acts as an immunogen in the rabbit. In summary, these data are extensions of the earlier results by Pereira and Kelly (1957), who at that time were able to demonstrate the persistence of adenovirus type 5 in the spleens of experimentally infected rabbits. However, these observations were not successfully repeated with the adenovirus serotypes 1, 2, 3 and 4 (Pereira and Kelly, 1957). Another example of human adenovirus type 5 persistence was shown by Duncan et al. (1978) who by employment of histological and immunofluorescence techniques demonstrated the de novo synthesis of structural proteins and of the early P-antigen as a consequence of an abortive infection of liver cells following intravenous injection of virus into mice.

The artificial host range in vitro for different types of human adenoviruses is known to vary considerably (Norrby et al., 1976). This may be exemplified by the ability of adenoviruses 2 and 5 to accomplish productive infections of hamster and rat embryo cells (Takahashi et al., 1966; Williams, 1973; Gallimore, 1974). On the other hand, the natural host range of adenoviruses is restricted to the normal host species (Norrby et al., 1976), but attempts of experimental infection in vivo with human adenovirus type 1 in cattle (Bettinotti and Straub, 1966) and in pigs (Betts et

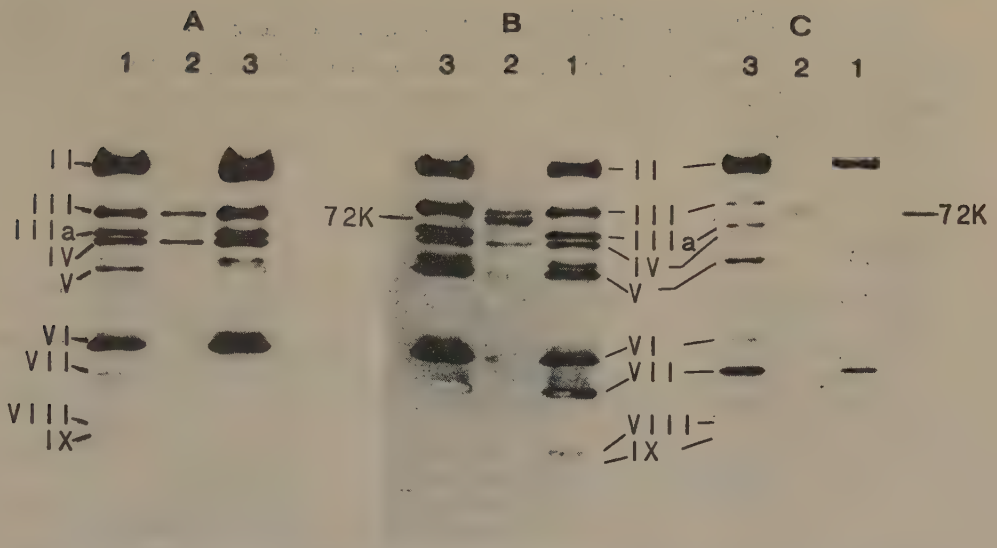

Fig. 3. Autoradiograms of immunoblotted SDS–polyacrylamide gels. Linear gradient SDS–polyacrylamide gels ranging from 6 to 20% acrylamide were electrophoresed at 50 V for 1 h and further at 100 V for 2.5 h. As primary antibodies 600 μl of either anti-intact Ad2 antiserum or anti-urea-dialyzed Ad2 antiserum were used and incubations were made at 37°C for 10 h. As the secondary antibody the IgG fraction of an anti-rabbit IgG antiserum from goat (Cappel, Cochranville, PA, U.S.A.) was used. This protein fraction was ^{125}I-labeled as described by Fraker and Speck (1978). The specific radioactivity of the labeled IgG was 2.57×10^8 cpm per mg of protein and material corresponding to approx. 4.4×10^6 cpm was added to each immunoblotting membrane. After incubations at 37°C for 12 h the membranes were washed and exposed to Kodak XG/14 X-ray films for 20 h at 20°C. Sample well No. 1 contains 7 μg of [^{35}S]methionine-labeled Ad2 virions; well No. 2 contains 5 μg of an unlabeled mixture of the 72K protein and pentons derived from a position corresponding to peak No. 5 of Fig. 1A in a DEAE-cellulose chromatogram; well No. 3 contains 20 μg of unlabeled Ad2 virions. As primary antibodies were used: Anti-urea-dialyzed Ad2 antiserum (gel A) and anti-intact Ad2 antiserum (gel B). In gel C a CBB-R stained gel from the SDS–PAGE separation is shown.

al., 1962) and type 5 in rabbits (Pereira and Kelly, 1957) and mice (Duncan et al., 1978) have been reported. Members of subgroup C of human adenoviruses (serotypes 1, 2, 5 and 6) are believed to establish latent or masked infections in lymphoid tissues (Flint, 1980) and tonsils (Wadell, 1984) of the natural host, and the finding of virus persistence in nonhomologous hosts may be a reflection of this phenomenon. Apart from the adenovirus 2-specific data presented in this communication the possibility should be considered, especially when using intact virions as immunogen, that semipermissiveness in vivo may exist in some virus–host systems normally regarded as nonpermissive.

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II.

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ASSESSMENT OF SPECIFIC VIRUS INFECTIVITY AND VIRUS NEUTRALIZATION BY A PROGENY VIRUS IMMUNOTITRATION METHOD AS EXEMPLIFIED IN AN ADENOVIRUS SYSTEM

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An alternative method for the determination of specific virus infectivity and quantitative measurement of inhibitory activity of antibodies was developed using an adenovirus system. HeLa cells in 37 ml suspension cultures of 1.5×10^7 cells were infected with purified adenovirus 2 (Ad2) at different multiplicities of infection. After appropriate incubations, total progeny virus was isolated by a one-step CsCl gradient sedimentation procedure. Recovered virions were disrupted in the presence of 5 M urea and directly quantitated by rocket immunoelectrophoresis against an anti-hexon-antiserum. One infectious unit (IU) was defined as the lowest amount of virions capable of producing maximum yield of progeny virus in the standardized system, and corresponded to 32 physical particles, which also equalled one plaque-forming unit (pfu). The coefficient of the inter-experimental variation of the total virus yield determinations was 13%.

Reduction in progeny virus synthesis was taken as a measurement of the degree of the inhibitory effect of neutralizing antibodies. A linear relationship was obtained between dilution of a neutralizing antiserum and reduction in synthesis of progeny virus. Separate determinations of such neutralization revealed a coefficient of variation of 5.5%.

immunotitration adenovirus neutralization specific infectivity

INTRODUCTION

The adenovirus system is well characterized with regard to regulation of gene expression (Philipson, 1979) and localization of the 10 to 12 structural proteins of the virion (Ginsberg, 1979). For infectivity titration of this virus the principal methods are the classical plaque titration assay (Dulbecco, 1952; Bonifas and Schlesinger, 1959; Kjellén, 1961) and the fluorescent cell-counting procedure (Coons and Kaplan, 1950; Wheelock and Tamm, 1961; Philipson, 1961). In some instances it is also desirable to assess the specific infectivity (physical particles per infectious unit) of a purified virus preparation, and for this purpose the number of physical particles is first determined by measurement of UV-absorption as described by Maizel et al. (1968a).

The aim of this investigation was to develop an infectivity and neutralization titration method employing cells in suspension culture which also provides results

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within 2 to 3 days. The procedure is based on rocket immunoelectrophoretic quantification of urea-disrupted progeny virions, released and isolated from small suspension cultures grown for each sample dilution. For the immunological analyses the hexon antigen was chosen, since this component constitutes around 50% of the total virion protein content (Maizel et al., 1968b). Moreover, for the purpose of antiserum production this antigen is easily obtained in large quantities and in a pure form from the cellular pool of the structural proteins synthesized in excess late in infection (Pettersson et al., 1967).

MATERIALS AND METHODS

Cells and viruses

HeLa cells were maintained in suspension cultures at densities of 2.5×10^5 to 5×10^5 cells per ml in Eagle's minimal essential medium (E-MEM) supplemented with 5% fetal calf serum and 5 µg/ml of gentamicin. Medium and supplements were obtained from Flow Laboratories Ltd., Irvine, Scotland. In some experiments cells were grown at densities between 2×10^5 and 3×10^5 or between 5×10^5 and 7×10^5 cells per ml (low and high cell density series, respectively). The wild type of human adenovirus 2 (Ad2) was propagated and purified as described previously (Everitt et al., 1977).

Infection of cells with Ad2 in the standardized assay

For the analysis of each sample 1.52×10^7 cells were sedimented and E-MEM was added to the cell pellet to give a final volume of 1 ml. Cells for each series were transferred to individual 100 ml screw-capped laboratory bottles (Schott Glaswerke, F.R.G.) containing 2 ml of E-MEM. Virus was diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and added at different multiplicities of infection (MOI) as indicated in the text. For attachment, the virus–cell mixtures were incubated at 37°C for 30 min on a rocker platform, whereafter 34 ml of E-MEM was added containing serum and gentamicin as above. The cells were further incubated at 37°C for 39 h on an orbital shaker platform with slow agitation.

Assessment of virus neutralization in the standardized assay

To 4.4×10^{10} virions in volumes of 25 µl were added 25 µl of a heat-inactivated anti-Ad2-antiserum at final dilutions as indicated in the text. PBS containing 1% BSA was used as diluent. The virus–antibody mixtures were incubated at 37°C for 30 min, whereafter 1,500 µl PBS containing 1% BSA were added. After vigorous mixing, virus–antibody complexes were added to 1.52×10^7 cells at calculated MOIs of one IU per cell as defined in the text. Virus–cell mixtures were further incubated as described above. In the appropriate control, the anti-Ad2-antiserum was omitted and replaced by PBS containing 1% BSA or by a pre-immune serum.

Recovery of progeny virus from infected cells in the standardized assay

At 39 h post-infection (p.i.) infected cells were sedimented (1,100 × *g* for 10 min) and quantitatively transferred to Ultra-clear centrifuge tubes (Beckman Instruments Inc., CA) by three washes of 0.33 ml each of 50 mM Tris–HCl buffer, pH 8.1. The cells were ultrasonically disintegrated for 3 × 7 s on ice whereafter *n*-butanol was added to a final concentration of 1% and the mixture was incubated on ice for 1 h (Everitt et al., 1977). Cell debris was removed by centrifugation at 10,000 × *g* for 15 min and the virus-containing supernatants were layered onto double CsCl cushions of 1.40 and 1.32 g/ml, 1.0 and 1.2 ml, respectively, in 4.2 ml polycarbonate centrifuge tubes (MSE Scientific Instruments Ltd., Sussex) and centrifuged at 83,000 × *g* for 2 h. After sedimentation, the contents of the tubes were fractionated from the bottom into 3 parts by an over-pressure device; fraction 1 contained the first 80 drops from the bottom; fraction 2 consisted of the next 20 drops comprising the virus material, and fraction 3 contained the remaining material in the tube constituting incomplete particles and soluble antigens.

Assessment of progeny virus by rocket immunoelectrophoresis (RIE)

An equal volume of freshly prepared 10 M urea (E. Merck, Darmstadt, F.R.G.) dissolved in 73 mM Tris–24 mM barbitone buffer, pH 8.6, containing 0.45 mM Ca-lactate (T-buffer) was added to each of the fractions obtained from the virus isolation step. After mixing on a Vortex Mixer, the samples were incubated at 20°C for 10 min and subsequently subjected to RIE (Laurell, 1966). Appropriate amounts of anti-hexon-antiserum were mixed with 1% agarose (Type 1, Sigma Chemical Co., St. Louis, MO) in T-buffer and cast onto 110 × 200 mm glass plates. Fraction 3 was immunoelectrophoresed in gels containing 2.5% anti-hexon-antiserum and fraction 2 in two parallel gels containing 0.25 and 0.5% of the same antiserum. For virus determinations in the latter case, the mean values between the two measurements were used. Aliquots of 5 µl were applied to each sample well and electrophoreses were performed at 75 V, for 15–18 h at 10°C. Gels were compressed, dried and stained in 0.2% Coomassie Brilliant Blue R dissolved in methanol–acetic acid–water (45 : 10 : 45).

Calibration curves

Known quantities of purified adenovirus in a CsCl solution of 1.345 g/ml were diluted in CsCl in T-buffer ($\rho = 1.345$ g/ml) whereafter equal volumes of 10 M urea in T-buffer were added. Aliquots of 5 µl were subjected to RIE as described above. Immunoelectrophoretic rocket heights, measured from the center of the wells, were plotted against the corresponding amounts of virus particles. Calculations of purified virus concentration in this report were based on a value of 1×10^{12} particles per optical density unit (Maizel et al., 1968a).

RESULTS AND DISCUSSION

Distribution of virus-specified material after ultracentrifugational separation

Fractions of 5 drops were collected to examine the distribution in a centrifuge tube of virus-specified material recovered after centrifugation at 83,000 × *g* for 2 h as described in Materials and Methods. Urea (10 M in T-buffer) was added to each fraction and aliquots were subjected to RIE against anti-hexon-antiserum as shown in Fig. 1.

The virus material (fractions 17–20, drops 81–100) appeared to be well separated from the soluble antigens and incomplete particles of fractions 21–46. Consequently the fractionation procedure was standardized for all experiments which follow in this report and three major fractions were subsequently collected as indicated at the bottom of Fig. 1.

Calibration curves

Based on the assumption that 1 optical density unit of virus equals 1×10^{12} particles, known amounts of purified adenovirus particles were diluted in either T-buffer or in CsCl ($\rho = 1.40$ g/ml) dissolved in Tris–HCl buffer, pH 8.1. Equal vols of 10 M urea in T-buffer were added to the samples and then subjected to RIE. The heights of the rockets were plotted against the corresponding quantities of virus particles. It is obvious that CsCl (line II in Fig. 2A) influenced the precipitation pattern, resulting in shorter rockets as compared with the situation where T-buffer was employed as the diluent (line I in Fig. 2A). To account for the CsCl effect in RIE and since virus particles were recovered from CsCl gradients in a density region of 1.34–1.35 g/ml, calibration curves were constructed in which known amounts of virus particles in CsCl

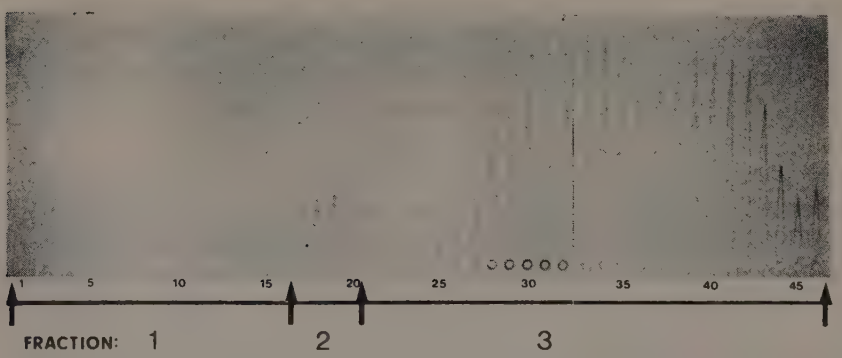

Fig. 1. Rocket immunoelectrophoretic analysis of the distribution of virus-specified material after separation by ultracentrifugation. Virus-infected cells were treated as described in Materials and Methods, and the contents of one centrifuge tube was fractionated into fractions of 5 drops, which after addition of equal volumes of 10 M urea in T-buffer, were subjected to RIE against 0.5% anti-hexon-antiserum. At the bottom of the figure the three major fractions are indicated which in all subsequent experiments were recovered.

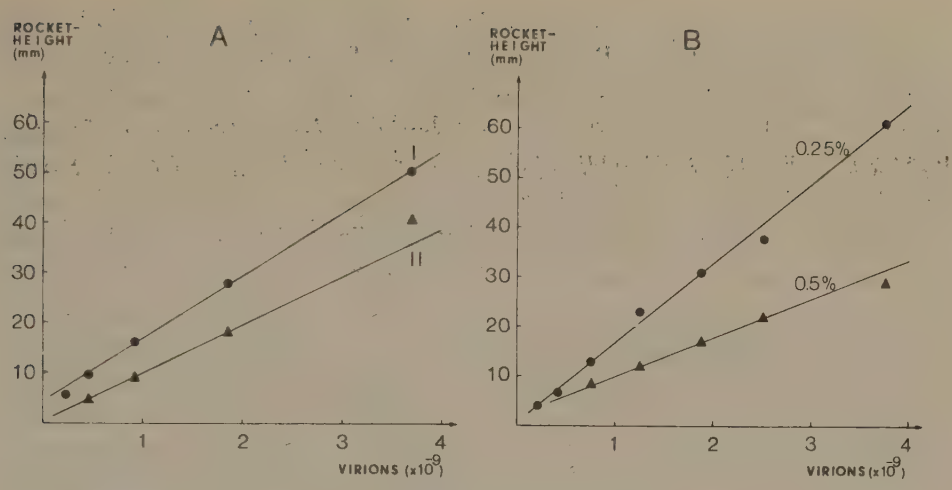

Fig. 2. Calibration curves. Panel A: The influence of CsCl on the rocket heights in the RIE analysis was studied when known amounts of purified virions were diluted in either T-buffer (line I) or in Tris–HCl-buffered CsCl of 1.40 g/ml (line II). The gel contained 0.5% anti-hexon-antiserum. Panel B: Known amounts of purified virions were diluted in T-buffered CsCl of 1.345 g/ml and subjected to RIE against two different concentrations of anti-hexon-antiserum as indicated.

of 1.345 g/ml were diluted in T-buffered CsCl of the same density, and subsequently added 10 M urea in the same buffer. RIE was performed in parallel gels containing 0.25 and 0.5% of anti-hexon-antiserum (Fig. 2B).

To evaluate further the calibration system, a known amount of virions was centrifuged on a double CsCl cushion at 83,000 × *g* for 2 h and fractionated into three portions as described above. Urea was added to fraction 2 which was thereafter subjected to RIE with the two concentrations of anti-hexon-antiserum. Five µl samples theoretically contained 1.97×10^9 virions and based on the calibration curves of Fig. 2B, the heights of the rockets produced values of 2.08×10^9 and 1.95×10^9 virions for the determinations using 0.25 and 0.5% of anti-hexon-antiserum, respectively. These results demonstrate that the ultracentrifugation procedure quantitatively recovered the virions of the applied sample and also indicated a good reproducibility of the RIE assay. With the antibody concentrations chosen, the detection limit was estimated to about 2×10^8 virions which corresponds to 56 ng of total virion protein (Maizel et al., 1968a) and 28 ng of hexon protein assuming that the hexon accounts for 50% of the total protein content of the virus particle (Maizel et al., 1968b). In the normal RIE analysis and in the absence of CsCl, the detection limit for the purified hexon antigen was 5–10 ng (not shown).

Progeny virus production in response to different MOIs

Cells were infected with Ad2 at different MOIs ranging from 1 to 2,000 particles per cell and at 39 h p.i. virus was isolated as described in Materials and Methods. Fractions

2 and 3 were recovered and to each was added an equal volume of 10 M urea in T-buffer and samples were subjected to RIE. The production of progeny virus in response to the different MOIs is shown in Fig. 3. Parallel analyses of the hexon antigen content of fraction 3 were routinely carried out as a technical control of the virus preparation procedure (not shown). At MOIs in the interval of 3–32 particles per cell, a linear relationship was obtained and the production of progeny virus gradually increased in this interval. A plateau was reached at an interpolated MOI value of 32 particles per cell, which was therefore defined as one IU. This point of the curve indicates that all susceptible cells at a MOI of 32 were infected by at least one IU. This assumption is based on the observation that no further increase in virus yield was obtained at higher MOIs (Fig. 3). However, at extreme MOIs, e.g. 5,000 particles per cell, the total virus yield was reduced by 68%. This is most probably a reflection of cellular damage at the stage of the early virus–cell interaction. Attempts to fit the data in Fig. 3 to a poissonian distribution and thereby calculate a value for virus yield per cell at different MOIs were not successful. This may be due to the existence of positive cooperativity in the adenovirus attachment system (Persson et al., 1983), which implies that the

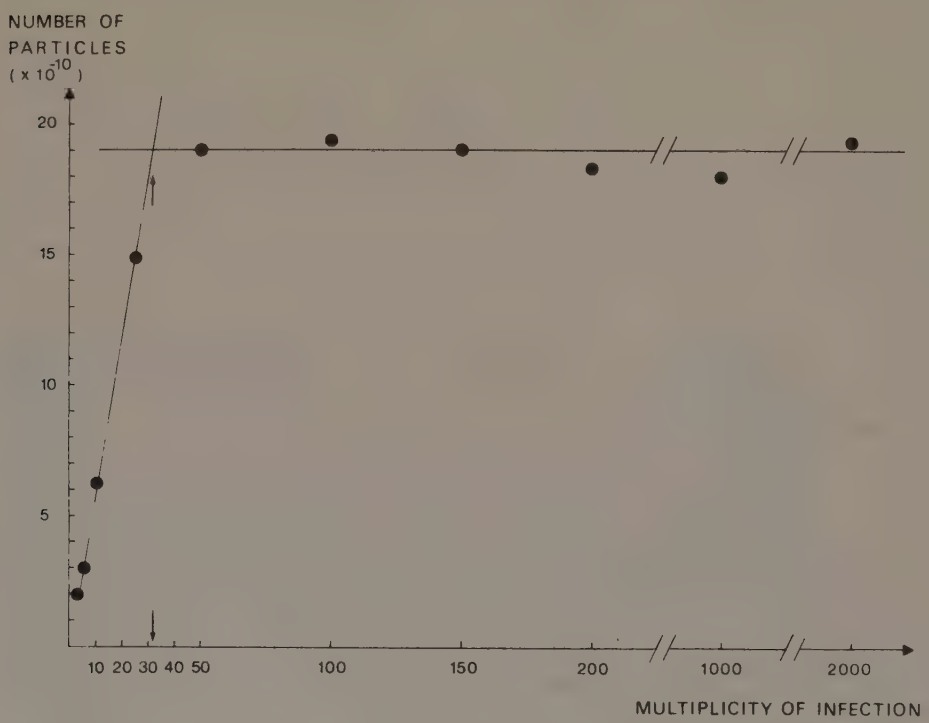

Fig. 3. Progeny virus production in response to different multiplicities of infection. Cells were infected with purified Ad2 at different multiplicities and incubated as described in Materials and Methods. Virus was isolated and quantified by RIE analysis. The amounts of progeny virus particles were obtained from the calibration curves shown in Fig. 2B, and plotted against the corresponding MOIs. The arrows indicate the interpolated intersection for maximum virus yield at the lowest input multiplicity = 1 IU.

attachment of the first virus particle promotes the binding of the second and thereby a true random binding of virions on cells is not at hand. In an earlier study by Russell et al. (1967) the accumulated antigen production was shown to be temporally multiplicity dependent. However, at an extended incubation period of 30 h p.i. all cells produced the same excess amount of structural proteins irrespective of employed multiplicity. In our standardized procedure the infected cells were harvested at 39 h p.i. and therefore it is assumed that differences in recovered virus yields only are reflections of the actual number of infected cells.

The specific infectivity of the virus preparation used in this investigation was thus estimated to be 32 virus particles per IU. For the same virus pool a comparison with the established plaque titration method, in two separate determinations (Kjellén, 1961), revealed a mean value of 35 physical particles per pfu. It is apparent that the titers of specific infectivity obtained with the present method (virions/IU) and those based on virions/pfu most likely mirror the same infectious entity.

The detection limit of progeny virus was obtained at a MOI of 3 particles per cell and a total of about 2×10^{10} virions were produced (Fig. 3). The final volume of fraction 2 was 570 µl after addition of urea and thus a 5 µl aliquot in the RIE assay contained about 1.8×10^{8} virions which should be compared with the detection limit of around 2×10^{8} virions as obtained from the calibration curves (Fig. 2B). One obvious way to increase the sensitivity of the assay would be to collect the virus material of fraction 2 in a smaller volume, which in this report was standardized to 20 drops, 285 µl, and thus increase the virus concentration. However, too narrow a fractionation might risk the recovery. The addition of solid urea instead of a 10 M solution and an increased number of cells are also apparent ways to increase the sensitivity of the assay.

There was a good correlation between the production of progeny virus and soluble hexon antigen at different MOIs, and 2–4% of the synthesized hexons in infected cells were assembled into mature virions in this system (data not shown).

Reproducibility of the assay

Fluctuations of the cellular status (biological variation) and differences in handling of the cells, virus isolation and measurements of progeny virus production (technical variation) are all factors which influence the reproducibility of the assay, and may be referred to as inter-experimental variation. The magnitude of this variation was estimated in a study performed over an 8 wk period where 9 separate cell cultures of 1.52×10^{7} cells were infected at a MOI of one IU per cell. The yield of progeny virus was determined as described in Materials and Methods, and a mean value of 2.18×10^{11} virions was obtained, with a coefficient of variation of 13% (Table 1).

When the present titration method is used to measure inhibitory effects on virus replication of e.g. drugs, reagents or antibodies, a control value of progeny virus production is set at 100%. This implies that variations in the system due to differences in the cellular status are eliminated and therefore the remaining variation of the assay will be referred to as the intra-experimental variation. Measurement of this variation

TABLE 1

Inter- and intra-experimental variations of the assay

Expt. no.	Progeny virus production[a] (control)	Neutralization[b] (% reduction)
1	2.02×10^{11}	N.D.[c]
2	2.04×10^{11}	N.D.
3	2.09×10^{11}	N.D.
4	1.86×10^{11}	N.D.
5	1.95×10^{11}	N.D.
6	2.12×10^{11}	62.2
7	2.35×10^{11}	66.7
8	2.75×10^{11}	70.2
9	2.47×10^{11}	63.4
Mean =	2.18 ± 0.285 (SD) $\times 10^{11}$	65.6 ± 3.59 (SD)

[a] In 9 separate experiments 1.52×10^7 cells were infected at a calculated MOI of 1 IU per cell and the progeny virus production was determined as described in Materials and Methods (inter-experimental variation).

[b] A neutralizing anti-Ad2-antiserum at a final dilution of 1/1,000 was mixed with Ad2 before addition to cells as described in Materials and Methods. Neutralization is given as a percentage reduction of progeny virus production as compared with the corresponding control value (intra-experimental variation).

[c] Not determined.

was performed in neutralization tests, in which an anti-Ad2-antiserum at a final dilution of 1/1,000 was allowed to react with virions before exposure to the cells. The amount of progeny virus production was compared with a control in which the neutralizing serum was omitted and replaced by either PBS containing 1% BSA or by a pre-immune serum. The same control value was obtained in these two situations. In Table 1 the degree of neutralization in four separate determinations is shown. A mean value for the neutralization was estimated to 65.6% with a coefficient of variation of 5.5%. When dilutions of an anti-Ad2-antiserum were analyzed in the standardized neutralization assay, a linear relationship was obtained between the serum dilutions and the corresponding values for neutralization (Fig. 4). The neutralization test works within the multiplicity interval 3–32 particles per cell (compare Fig. 3), i.e. a maximum value of 90% neutralization is achieved due to the detection limit of 3 particles per cell. This would be enough for many purposes, but in some cases it is desirable to achieve a value for even higher degrees of neutralization. In those cases one could increase the sensitivity of the assay as described above or increase the MOIs; for every doubling of the MOI, the remaining interval up to the 100% level is reduced by half and asymptotically reaches a value of 100%.

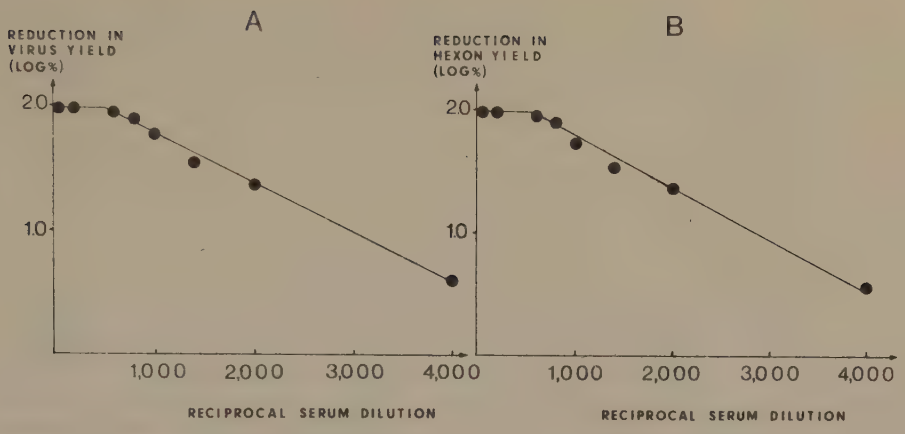

Fig. 4. Neutralization of adenovirus infectivity. Dilutions of an anti-Ad2-antiserum were mixed with Ad2 before exposure to the cells. In the figure the final dilutions of antiserum in the antibody–virus mixtures are shown. After incubation of the cells, preparation of the progeny virus and RIE analysis, the amounts of progeny virus were obtained from the calibration curves shown in Fig. 2B. Neutralization is given as a percentage reduction in progeny virus yield when compared with the control in which the neutralizing antiserum was replaced by PBS containing 1% BSA (panel A). In panel B the corresponding reduction in accumulated hexon antigen is shown and measured as reduction in rocket heights as compared with the control.

Technical considerations

The influence on progeny virus yield of the cell concentration in the stock cell culture was studied. Low- and high-cell density series were used as described in Materials and Methods. After 1 wk of cell cultivation, the high density series appeared unhealthy with many dead cells and therefore this series was not considered for further experiments. Cells from the low density culture were, however, subjected to virus infection at MOIs of 1 and 0.5 IU per cell. As compared with the control cell culture, maintained at a cell density of 2.5×10^5 to 5×10^5 cells/ml, the amount of the recovered virus in the low density series was reduced by 19.1% and 18.6% for the two MOIs of 1 and 0.5, respectively.

The accumulated virus production as a function of incubation time p.i. was also examined in a separate experiment. Cells were infected with Ad2 at a MOI of one IU per cell as described in Materials and Methods, and incubated at 37°C for 33, 36, 39, 42, 45 and 58 h. The total virus yield was determined for each time, and no difference was revealed between 33 and 45 h, but at 58 h a reduction of 34% was observed.

The efficiency of virus disintegration in the presence of urea was analyzed by RIE. Material obtained from fraction 2 (the virus material) and fraction 3 (the soluble antigens) was divided into series of 9 tubes each, and equal volumes of urea in T-buffer were added to yield final concentrations of 1, 1.5, 2, 2.5, 3, 3.5, 4, and 5 M. It was apparent that the immunoprecipitation of the hexon antigen (fraction 3) in the RIE assay was not affected by the presence of urea (Fig. 5C). On the other hand, no distinct

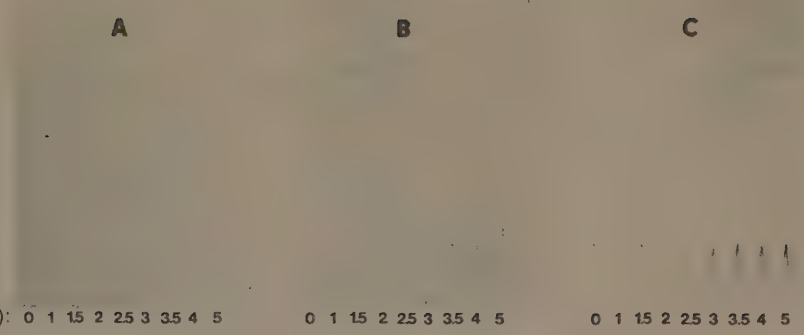

Fig. 5. Influence of urea concentration on rocket formation. Virus material (fraction 2 in Fig. 1) and soluble antigens (fraction 3 in Fig. 1) were divided into 9 tubes each, added urea at different concentrations as indicated in the figure and subjected to RIE. Virus containing fractions were electrophoresed in a gel with 0.25% (gel A) or 0.5% (gel B) of anti-hexon-antiserum. The soluble antigens were analyzed in a gel containing 2.5% of the same antiserum (gel C).

rockets were formed when urea was added to the virus material of fraction 2 at final concentrations of 1 M or less. The sharpness of the precipitates and the rocket heights gradually increased when the final urea concentration was raised between 1.5 and 5 M (Fig. 5A and B). At a temperature of 37°C and in the presence of 5 M of urea a prolonged incubation period of 30 min did not change the precipitation patterns or the rocket heights as compared with incubations at 20°C for 10 min (data not shown).

We consider that the present immunotitration method offers some advantages as compared with the established plaque titration techniques. The plaque titration methods for slowly released viruses, such as adenoviruses, all suffer from prolonged incubation periods and the number of plaques increase for 2 to 3 wk, with the concomitant risk of cell monolayer disruptions (Kjellén, 1961). The present procedure therefore offers a less time consuming alternative to those methods, and based on statistical evaluations the present technique has good reproducibility, comparable with the 20% precision for the pfu assay (Philipson, 1961; Bishop and Koch, 1969).

There was a good correlation between the progeny virus- and accumulated hexon synthesis in infected cells (Fig. 4), and since both are examined in the RIE assay, any misjudgement due for example to technical error is reduced. The fact that suspension cultures are employed for the immunotitration assay, provides experimental advantages when handling cells treated with drugs or other reagents, and also in terms of producing statistically controlled infections.

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Interaction Between HeLa Cells and Adenovirus Type 2 Virions Neutralized by Different Antisera

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Three adenovirus type 2-specified immunogens elicited neutralizing antibodies when injected into rabbits; these were the fiber, the hexon, and the penton base. Adenovirus type 2 virions, neutralized by antihexon- or anti-penton base antisera, attached to HeLa cells to the same extent as untreated control virus, and after attachment, neutralized viruses also became sensitive to DNase treatment. A fraction of 75 to 80% of the attached antibody-treated virions penetrated the plasma membrane, which should be compared with an 84 to 88% penetration level in the control series. A majority of the antihexon-neutralized virions was found in intracellular vesicles, as revealed with an electron microscope, but in the case of anti-penton base neutralization, a maximum of 50% of the virions was retained within vesicles, and ca. 30% was free in the cytoplasmic compartment. A value greater than 45% was never obtained for neutralization with a monospecific anti-penton base antiserum, which could imply the existence of alternative pathways for virus penetration into HeLa cells—one of these being sensitive to treatment with anti-penton base antiserum. Antisera containing antifiber specificities efficiently aggregated virions, and the aggregation data mirrored the degree of neutralization. Antifiber-neutralized virions attached to cells to a three- to five times greater extent than untreated control virus, but the former virions had a reduced ability to become sensitive to DNase treatment. Around 15% of the attached antifiber-treated virions was found as large aggregates inside multivesicular bodies or lysosomes.

Antibody-mediated neutralization of viruses has been extensively studied and has been reviewed by Mandel (20, 21) and Dimmock (4). In the adenovirus system, the hexon antigen from serotypes 1 to 5 has been shown to elicit neutralizing antibodies when immunized into different host species (1, 9, 14, 22, 24, 25, 35, 40, 42, 43), although the neutralizing potency of those antisera varies among the serotypes. Antisera against the fiber antigen of serotypes 1, 2, and 5 have been reported both to contain (1, 22, 42) and to lack (14, 27, 28, 40) significant amounts of neutralizing antibodies. One possible explanation for these conflicting data could be the employment of different neutralization assays by these investigators. An antifiber antiserum effectively aggregates virions (5, 31, 39), and aggregation of virions in vivo could be an obvious way to abolish virus infectivity, but this is not always considered as being equivalent to a true neutralization (20). Neutralization of adenovirus is a serotype-specific phenomenon; i.e., different serotypes are neutralized by homologous antisera (22, 24, 41, 42). Heterologous neutralization is achieved among some serotypes, provided that the antibodies are allowed to attach to virions at low pH values (14); however, isolated type-specific antihexon antibodies exert a strong neutralizing effect (9, 43, 44), whereas the group-specific antihexon antibodies do not (9, 43). Antisera against the penton base and protein IIIa have been reported not to be involved in neutralization of adenovirus type 2 (Ad2) (2, 16).

The aims of the present investigation were to evaluate the role of different viral immunogens in eliciting neutralizing antibodies and to study the subsequent interaction between neutralized virions and HeLa cells. For this purpose, two series of antisera were used. One was a total anti-Ad2 antiserum from which antifiber or antihexon antibodies or both were removed, and the other was a series of monospecific antisera produced in response to extensively purified Ad2 structural proteins. Virions neutralized by various antisera were subsequently studied with regard to patterns of attachment, penetration, uncoating, and cellular distribution.

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MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at densities between 2.5×10^5 to 5×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μg of gentamicin per ml. For attachment studies, cells were grown in petri dishes (60-mm diameter) in Eagle minimal essential medium formulated for monolayer cultures and supplemented with 10% fetal calf serum and gentamicin as above.

The wild type of human Ad2 was propagated and purified as previously described (6, 8). [^{3}H]thymidine-labeled virus was prepared by the method of Svensson et al. (38).

Production of antisera. (i) Polyspecific antisera. Antisera against Ad2 were obtained after three successive intramuscular immunizations in rabbits over a 4-week period (10) with 1.4×10^{12} highly purified virions each time, corresponding to 0.36 mg of total protein (17). The immunogens were mixed with equal volumes of Freund complete adjuvant at the first and second immunization. The rabbits were bled 1 week after the last immunization and thereafter weekly over a 4-week period. Two separately prepared antisera against intact Ad2 particles were used in this investigation, and these will be referred to as no. 111 and no. 113.

(ii) Monospecific antisera. The major soluble antigens (i.e., hexon, fiber, and penton base) remaining after virus isolation by preparative ultracentrifugation on CsCl gradients were separated by DEAE-cellulose chromatography as described by Pettersson et al. (26) and modified as recently described (45). The hexon antigen was further purified by quaternary aminoethyl-Sephadex chromatography and negative affinity on a column of antipenton-immunoglobulin G-Sepharose 4B (see below). The fiber antigen was further purified by

carboxymethyl-Sephadex chromatography, and the penton base was purified by quaternary aminoethyl-Sephadex chromatography, followed by negative affinity on antifiber-immunoglobulin G-Sepharose 4B and antihexon-immunoglobulin G-Sepharose 4B. Purified protein IIIa, prepared as described by Everitt and Philipson (7), was obtained from L. Philipson.

Rabbits were immunized with each protein (20 to 50 μg) according to the schedule described above, and preimmune sera were collected before all immunizations. Each monospecific antiserum was titrated by rocket immunoelectrophoresis against the anti-Ad2 antiserum no. 113 and subsequently diluted with phosphate-buffered saline (PBS) to contain the same concentration of precipitating antibody against the appropriate antigen. All antisera were heat inactivated at 56°C for 30 min before use.

Cyanogen bromide activation of Sepharose and coupling of antigens. Sepharose 4B in portions of 3 g was activated by addition of CNBr dissolved in bidistilled water. Either 14 mg of hexon or 4 mg of fiber antigen, both purified as described above, was added to the activated gel. Incubations and blocking of remaining reactive groups were performed as described by the manufacturer of Sepharose 4B. The immunoglobulin fractions from monospecific antisera were precipitated in $(NH_4)_2SO_4$ at a concentration of 33% relative saturation and were coupled in an analogous way.

Removal of selected antibody specificities from an anti-Ad2 antiserum. Portions (200 μl) of an anti-Ad2 antiserum (no. 111) were passed through columns of fiber-Sepharose 4B or hexon-Sepharose 4B. All UV-absorbing material above 0.01 at 280 nm not retained by the Sepharose column was collected and dialyzed extensively against bidistilled water. After lyophilization, the absorbed antisera were resuspended in PBS to give four times the original volume. The specificities of the immunoabsorbed antisera were tested by rocket immunoelectrophoresis (RIE) (15).

RIE. Appropriate amounts of antisera, as indicated in Fig. 1, were mixed with 1% agarose in 73 mM Tris–24 mM barbitone buffer (pH 8.6) containing 0.45 mM calcium lactate. Portions (5 μl) were applied in each sample well, and electrophoreses were performed at 75 V for 16 to 18 h at 10°C. After electrophoreses, the gels were compressed, dried, and stained in 0.2% Coomassie brilliant blue R dissolved in methanol-acetic acid-water (45:10:45).

Immunoaggregation of virions by different antisera. Samples (25 μl) of [^{3}H]thymidine-labeled virions (4.4×10^{10} particles and 125,000 cpm of radioactivity) were added to an equal volume of the appropriate antiserum, and when necessary, these were diluted in PBS containing 1% bovine serum albumin (BSA) to yield final antiserum dilutions ranging from 1/2 to 1/4,000. Higher concentrations of antisera were obtained by adding 100 μl of the appropriate antiserum to 25 μl of virus, and these series will be referred to as the 1/1.25 samples.

After incubations at 37°C for 30 min, 50 μl of PBS was added to the virus-antibody mixtures, and after thorough mixing, the samples were layered onto sucrose gradients formed on top of a cushion of CsCl (1.4 g/ml) and consisting of 2 × 1.5 ml of 10 to 25% (wt/vol) sucrose in 5 mM Tris hydrochloride–0.2 mM EDTA (pH 7.5). Centrifugations were performed at 50,000 × *g* for 10 min at 5°C. The tubes were fractionated from the bottom into fractions of 5 drops each, and portions were assayed for radioactivity in Ready-Solv (Beckman Instruments AB) after precipitation with trichloroacetic acid on paper filter disks. For each sample, the immunoaggregated radioactivity recovered on the CsCl cushion was monitored relative to the rest of the radioactivity in the tube.

Neutralization of virus infectivity measured by progeny virus immunotitration. The neutralization tests were performed by a progeny virus immunotitration assay as recently described (46). Briefly, 4.4×10^{10} virions in volumes of 25 μl were added to appropriate antisera at final dilutions as described above. After incubations at 37°C for 30 min, PBS containing 1% BSA was added to each sample to give a final volume of 1,550 μl. Samples (25 μl) of antibody-virus complexes were added to 3 ml of Eagle minimal essential medium containing 1.52×10^7 cells in 100-ml screw-cap Schott Glaswerke laboratory bottles, yielding a multiplicity of infection of 46 particles per cell, equivalent to one infectious unit per cell. After attachment for 30 min at 37°C, 34 ml of Eagle minimal essential medium containing serum was added to each bottle, yielding a concentration of around 4.1×10^5 cells per ml. At 39 h postinfection, progeny virus was prepared by a one-step CsCl gradient centrifugation procedure. An equal volume of 10 M urea was added to the virus material, and progeny virus was quantified by RIE against an antihexon antiserum. Neutralized virus samples were compared with separate controls in which the neutralizing antisera were replaced by PBS containing 1% BSA or preimmune serum.

In vitro DNase sensitivity of virions after incubation with different antisera. The DNase sensitivity analyses, both in vitro and of virions attached to cells (see below), were performed essentially as described by Joklik (11). Appropriate antisera at the dilutions described above were added, in volumes of 25 μl, to 4.4×10^{10} [^{3}H]thymidine-labeled virions. After incubations at 37°C for 30 min, 1 volume of 1% BSA in PBS was added to the sample, whereafter 7.5×10^9 particles were withdrawn and incubated for 30 min at 37°C in the presence of 0.2 mg of DNase I and 7.5 mM $MgCl_2$ in PBS in a final volume of 60 μl. The samples were subsequently diluted with 400 μl of PBS containing 0.1% BSA, and undegraded [^{3}H]DNA was precipitated with trichloroacetic acid at a final concentration of 10%. Trichloroacetic acid precipitates were pelleted, washed with ethanol, and dissolved in Protosol (New England Nuclear). The supernatants and the precipitates were subsequently assayed for radioactivity in 0.4% Omnifluor (New England Nuclear) in toluene-methanol (1:1).

Attachment of virus-antiserum mixtures. Confluent monolayers of HeLa cells were washed with PBS containing 1% BSA. [^{3}H]thymidine-labeled virions were preincubated with the appropriate antiserum for 30 min at 37°C as described above and added to the monolayer cells at multiplicities of infection of 2,000 in total volumes of 500 μl of Eagle minimal essential medium formulated for monolayer cultures. Incubations were performed for 60 min at 37°C on a rocking platform. Unattached virions were aspirated, and the cells were washed once with 1% BSA in PBS and once with 0.02% EDTA in PBS. The monolayers were incubated in the presence of the latter solution at 37°C until the cells were released. The radioactivity of the cellular fraction and the washing solutions was measured, and the percentage of virus attached was calculated.

DNase sensitivity of virus-antiserum complexes attached to cells. [^{3}H]thymidine-labeled virions, preincubated with different antisera, were allowed to attach to cells in monolayer cultures for 60 min at 37°C as described above. The cells were released from the petri dishes as described above, transferred to centrifuge tubes, and sedimented. Suspension of cells was done in 10 mM sodium phosphate buffer (pH 7.3)

containing 10 mM $MgCl_2$. The cells were disrupted by addition of sodium deoxycholate to give a concentration of 0.5%. After addition of 1 mg of DNase I and incubation at 37°C for 30 min, the undegraded DNA was precipitated with trichloroacetic acid at a final concentration of 10%. Precipitates were pelleted, washed with 70% ethanol, dissolved in Protosol, and assayed for radioactivity.

Electron microscopy. Cells in suspension cultures were sedimented, washed once, and suspended in PBS at a concentration of 5×10^7 cells per ml. Virions, pretreated with the appropriate antisera at concentrations indicated in Table 1, were added to the cells at calculated multiplicities of infection of 5,000. The samples were incubated for 60 min at 37°C, whereafter the cells were washed twice in 10 ml of PBS each time. The cells were resuspended in PBS and kept at 3°C at a density of 10^6 cells per ml. Cells were fixed in 2% glutaraldehyde for 30 min at 3°C in a roller bottle, after which the cells were washed three times in PBS. Postfixation with osmium tetroxide and dehydrations were performed essentially as described by Ryter and Kellenberger (32), and finally the cells were embedded in Epon.

Chemicals. Eagle minimal essential medium for suspension cultures and for monolayer cultures, fetal calf serum, and gentamicin were obtained from Flow Laboratories Ltd., Irvine, Scotland. Freund complete adjuvant was from Difco Laboratories, Detroit, Mich. DEAE-cellulose (DE 52) was obtained from Whatman Inc., Clifton, N.J., and Sepharose 4B was from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Agarose (type 1) and DNase I were obtained from Sigma Chemical Co., St. Louis, Mo. Ready-Solv HP/b was purchased from Beckman Instruments AB, Bromma, Sweden. Omnifluor and Protosol were obtained from New England Nuclear, Dreieich, Federal Republic of Germany. Sodium deoxycholate was from BDH Chemicals Ltd., Poole, England, and the laboratory bottles were from Schott Glaswerke, Mainz, Federal Republic of Germany.

RESULTS

Specificity of the immunoabsorbed antisera. Columns of fiber-Sepharose 4B and hexon-Sepharose 4B were used to selectively remove antifiber or antihexon antibodies from an anti-Ad2 serum (no. 111). The remaining specificities of the antisera were analyzed by RIE against different virion-specific antigens (Fig. 1). As compared with the control serum (Fig. 1A and B, gel 1), it was shown that the antibody titers against the penton base (Fig. 1A) and protein IIIa (Fig. 1B) were not significantly affected by the removal of antihexon antibodies (gel 2), antifiber antibodies (gel 3) or both (gel 4). Moreover, no precipitates against the homologous antigens could be detected among the absorbed antisera when they were tested by RIE against a wide range of concentrations of both antigens and antisera as exemplified in Fig. 1.

Immunoaggregation and neutralization of virions by different antisera. Two series of antisera were used. One was a total anti-Ad2 serum (no. 111) together with the absorbed antisera derived from the same serum pool (described above), and the other was a series of monospecific antisera, titrated in RIE and diluted to contain the same concentration of precipitating antibody as a reference anti-Ad2 serum (no. 113). Such immunoelectrophoretic titrations were necessary to compare the degree of aggregation and neutralization in the subsequent studies.

Virions were mixed with appropriate heat-inactivated antisera as described in Materials and Methods. Such mixtures were both analyzed for possible aggregation by centrifugational rate separation in sucrose gradients and assayed for neutralization as described. The aggregation patterns (Fig. 2, solid bars) mirrored the neutralization data (Fig. 2, open bars); i.e., a high degree of aggregation yielded a correspondingly high degree of neutralization. The most striking effects were observed with antisera containing antifiber specificities, i.e., the two anti-Ad2 sera (no. 111 and 113), the total anti-Ad2 serum (no. 111) minus antihexon antibodies, and the monospecific antifiber serum. For these four antisera, a final dilution of 1/200 displayed almost 100% neutralization and 100% aggregation (Fig. 2). The total anti-Ad2 serum minus antifiber antibodies revealed close to 100% neutralization at a final serum dilution of 1/8, and the antihexon serum revealed 80% neutralization and 30% aggregation at a final dilution of 1/2. When both antifiber and antihexon antibodies were removed from the total anti-Ad2 serum, neutralization of around 85% was observed at a final dilution of 1/1.25. This effect was probably due to the anti-penton base antibodies, since a monospecific anti-penton base serum displayed 45% neutralization at the same final dilution. A notable feature of the neutralization tests with the anti-penton base serum was the fact that no further increase in neutralization was achieved at higher serum concentrations; thus, a final serum dilution of 1/1.1 gave the same result as a 1/1.25 dilution (not shown). Preimmune sera and an anti-protein IIIa serum were both totally devoid of antibodies causing aggregation or neutralization.

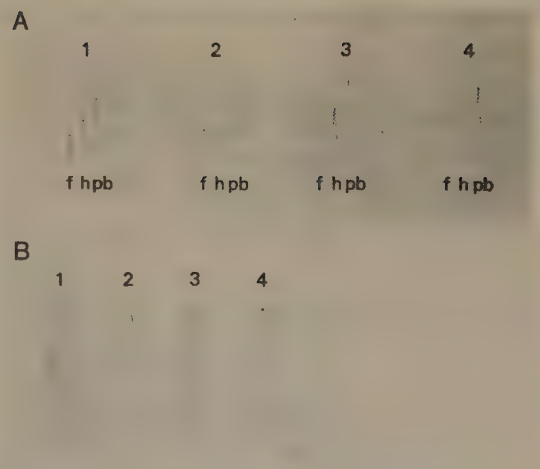

FIG. 1. RIE showing the specificities of the immunoabsorbed antisera. The agarose gels contained the following antisera: 1, Anti-Ad2 serum (no. 111); 2, Anti-Ad2 serum minus antihexon antibodies; 3, Anti-Ad2 serum minus antifiber antibodies; 4, Anti-Ad2 serum minus antihexon and antifiber antibodies. (A) Gels containing 0.5% of each antiserum. f, Fiber antigen; h, hexon antigen, and pb, penton base antigen. (B) Gels containing 2% of each antiserum. Protein IIIa was applied in all wells.

In summary, three classes of antibodies showed inhibitory effects on virus infectivity; these were antifiber, antihexon, and anti-penton base antibodies.

To exclude the possibility that the results above were due to formation of cytotoxic antibody-virion complexes (12, 13), with subsequent killing of the cells, the viability of the cells was studied by trypan blue exclusion. It was shown that the anti-Ad2 serum no. 113 at final dilutions ranging from 1/2 to 1/200 and the antihexon serum at a dilution of 1/1.25, i.e.,

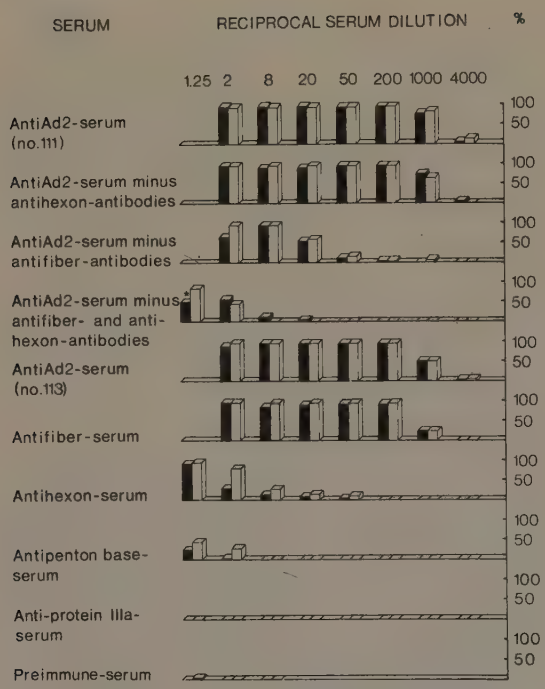

FIG. 2. Neutralization and aggregation of virions by different antisera. Ad2 virions were mixed with different antisera as indicated in the figure, and the mixtures were analyzed for aggregation in sucrose gradients (solid bars) and for neutralization by the progeny virus immunotitration method (open bars). The figures refer to the percentage of reduction in progeny virus yield as compared with that in the untreated control and to the percentage of the total applied radioactivity, subsequently recovered on a cushion of CsCl in sucrose gradients, for the neutralization and aggregation studies, respectively. In one aggregation sample (*), the reciprocal serum dilution was 1.5.

dilutions with maximal neutralization, neither caused increased trypan blue uptake of the cells, measured at 40 h postinfection, nor affected the proliferation of the cells during the infection period. However, at final anti-Ad2 serum dilutions of 1/1,000 to 1/4,000, in which the degree of neutralization is diminished, the frequency of stained cells at 40 h postinfection was 11 and 27%, respectively. In the control infection without neutralizing antiserum, 35% of the cells did not exclude the stain at 40 h postinfection. Antipenton base serum at final dilutions of 1/1.25 and 1/1.1 revealed intermediate effects with around 12% trypan blue-stained cells, which is in good agreement with the maximal neutralizing capacity of 45% displayed by this antiserum.

Antibody-mediated destabilization of virions in vitro. The possibility that incubations of Ad2 with various antisera could destabilize the virions in vitro, thus rendering the genome sensitive to DNase treatment, was investigated. Virions were mixed with the appropriate antisera at the same final concentrations as described above, and samples were subsequently analyzed for DNase sensitivity. After virus treatment with antisera containing antifiber specificities, i.e., total anti-Ad2 serum, anti-Ad2 serum minus antihexon antibodies, and antifiber serum, 15 to 20% of the labeled virion material became sensitive to DNase treatment at final serum dilutions of 1/8 (data not shown). A less pronounced tendency to destabilize was observed at both higher and lower dilutions of all antifiber-containing antisera. These observations could reflect the existence of a critical ratio between antifiber antibodies and virions. However, it seems reasonable to believe that these effects are not of any major significance for the capacity of these antisera to neutralize virus infectivity. No other antisera or preimmune serum displayed an effect on viral DNase sensitivity.

Attachment of virus-antibody complexes to HeLa cells. Attachment of the total mixtures of virus particles incubated with appropriate antisera was analyzed on monolayer cells and compared with a control in which virus was preincubated with either 1% BSA in PBS or preimmune serum. When virions were treated with any of the antifiber-containing antisera at various dilutions in a range of 1/8 to 1/1,000, an increase of a factor of three to five was observed in virion attachment, as compared with the control series (Fig. 3). The amount of virions attached in the presence of these antisera decreased and approached that of the control level when the serum concentrations were lowered from 1/1,000 to 1/4,000 (Fig. 3). The attachment of virions preincubated with antihexon serum, anti-penton base serum, or anti-Ad2 serum minus both antifiber and antihexon antibodies did not significantly differ from the results obtained after incubations with preimmune serum (Fig. 3). The anti-Ad2 serum minus antifiber antibodies did not influence the attachment except at the highest serum concentration.

DNase sensitivity of virus-antibody complexes attached to cells. In a previous report, it was suggested that virion destabilization takes place at the cell surface (37). To exam-

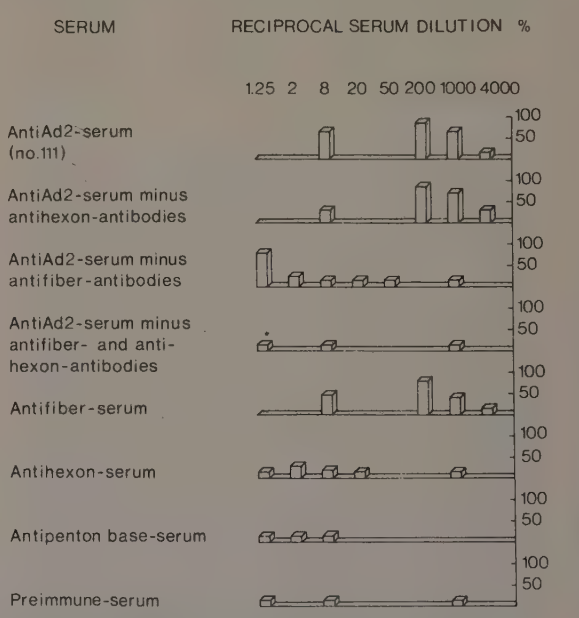

FIG. 3. Attachment of Ad2 virion-antibody complexes to cells. [^{3}H]thymidine-labeled virions were preincubated with different antisera and allowed to attach to monolayer cells for 60 min at 37°C. Cells were harvested, and the relative amount of attached virions was calculated after radioactivity measurement as described in the text. The absence of a box indicates that the relevant serum dilution was not tested. The figures refer to the percentage of attached virions. In one sample (*), the reciprocal serum dilution was 1.5.

ine whether pretreatment of virions with various antibodies affected the normal uncoating in any way, virus incubated with various antisera were allowed to attach to monolayer cells, and attached virions were subjected to DNase treatment as described in Materials and Methods. The background level of DNase sensitivity in vitro, as discussed above, was subtracted from the values obtained after the interaction of virus-antibody complexes with cells. All antifiber-containing antisera displayed a pronounced inhibitory effect on the cell-mediated destabilization of the virus particles (Fig. 4), whereas antihexon- or anti-penton base-neutralized virions became destabilized to the same extent as the untreated control. However, virions pretreated with anti-Ad2 serum minus antifiber antibodies at a reciprocal serum dilution of 1.5 were not destabilized to the same extent as the control.

Fate of antibody-treated virions attached to cells. In analogy with a previous study on virion internalization (37), ^{125}I-labeled anti-rabbit immunoglobulin G was used to quantify the degree of internalization of attached virion-antibody complexes. In the presence of either antihexon or anti-penton base sera (diluted 1/1.25), ca. 85% of the virions were internalized. This figure was reduced by half when an antifiber serum (diluted 1/200) was tested (data not shown). To follow further the cellular distribution of virions treated with various antisera, an electron microscopic investigation was performed (Table 1). After treatment of virions with a total anti-Ad2 serum or an antifiber serum, ca. 85% of the virions remained on the surface. The 15% of the virions which were internalized appeared in large aggregates and were located in vesicular structures, such as multivesicular bodies or lysosomes (Fig. 5A). Virions preincubated with an antihexon serum or an anti-penton base serum entered the cells nearly as efficiently as virions treated with a preimmune serum (Table 1). In the case of the antihexon serum, ca. 70% of the virions were accumulated inside vesicles, and only 8% were found near the nucleus. After pretreatment with anti-penton base serum, around 50% of the virions were left inside vesicles, which usually contained only a few virus particles, as also was the case for vesicles harboring antihexon-treated virions (Fig. 5B and C). However, 25% of the anti-penton base-treated virions appeared near the nuclear membrane, compared with the control series in which ca. 55% of the virions reached the nucleus (Table 1 and Fig. 5D).

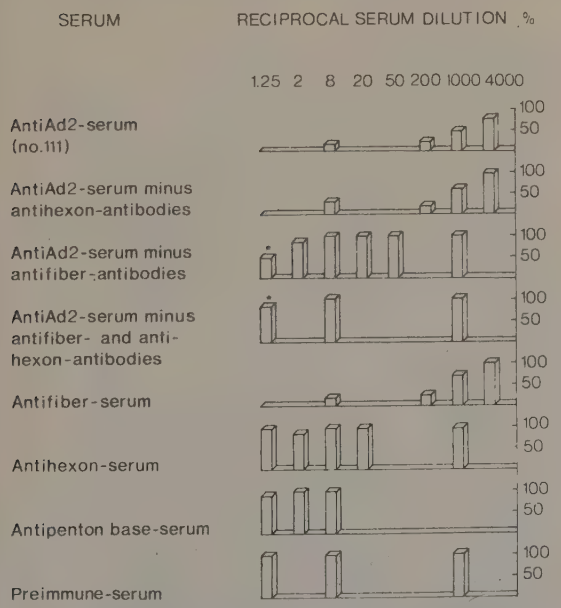

FIG. 4. DNase sensitivity of virion-antibody complexes attached to cells. [^{3}H]thymidine-labeled virions treated with different antisera were attached to monolayer cells for 60 min at 37°C. The cells were harvested, and the DNase sensitivity of the parental virion DNA was estimated as described in the text. The absence of a box indicates that the relevant serum dilution was not tested. The figures refer to the relative DNase sensitivity of the viral genome compared with the maximum uncoating efficiency of ca. 80% in a control sample of untreated virions and cells. In two samples (*), the reciprocal serum dilution was 1.5.

TABLE 1. Cellular distribution of Ad2 particles after incubation with various antisera

Serum treatment[a]	Relative distribution of Ad2 particles (%)[b]			
	Cell surface	Vesicles	Free	
			In cytoplasm	Near nucleus
Anti-Ad2 no. 113 (1/200)	88	12	0	0
Antifiber (1/200)	85	15	0	0
Antihexon (1/1.25)	21	69	2	8
Anti-penton base (1/1.25)	25	47	3	25
Preimmune (1/1.25)	16	16	12	56
PBS	12	14	20	54

[a] The figures within parentheses refer to the final dilution of antiserum in the reaction mixture.

[b] The relative distribution of ca. 200 counted virions within ca. 40 cells was calculated.

DISCUSSION

In this communication, we have demonstrated that three different viral immunogens elicit neutralizing antibodies as judged by reduction in progeny virus yield. A monospecific antifiber serum displayed a neutralizing capacity of the same magnitude as a total anti-Ad2 serum. When the antifiber antibodies were removed from an anti-Ad2 serum, the remaining neutralizing potential was of the same order as that of an antihexon serum, and when the anti-Ad2 serum was depleted of both antifiber and antihexon antibodies, the residual neutralizing activity equalled that of an anti-penton base serum.

A notable feature of the neutralization assays was the observation, especially among the antifiber-containing antisera, that the extent of neutralization paralleled the aggregation patterns. This raises the question whether the present neutralization data simply reflect the degrees of aggregation of virions, i.e., aggregation of several infectious units into a single infectious entity, which subsequently leads to a lower number of cells being infected than expected in the immunotitration assay. If this were true, addition of more than one infectious unit per cell would increase the total virus yield in the immunotitration assays. We tried this hypothesis among the different antisera by adding up to 250 neutralized infectious units per cell, but no increase in virus yield was observed.

Despite the aggregation of virions with antifiber-containing antisera, such complexes had a capacity to attach to cells at an even greater extent than untreated virions. An

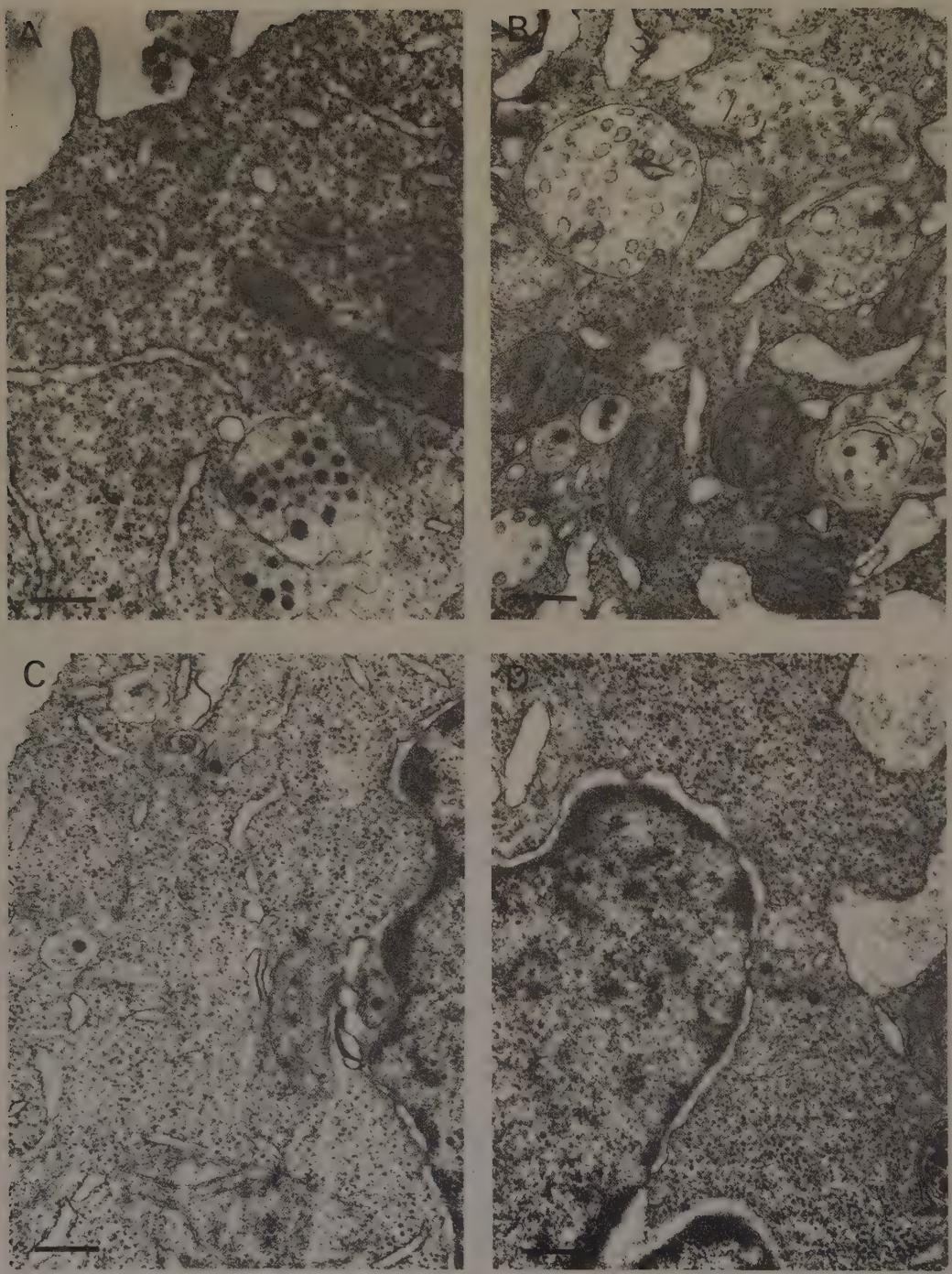

FIG. 5. Cellular distribution of antibody-treated adenovirus as observed with the electron microscope. Virions, pretreated with different antisera, were incubated with cells for 60 min at 37°C. Cells had virions pretreated with antifiber serum, dilution 1/200 (A); antihexon serum, dilution 1/1.25 (B); anti-penton base serum, dilution 1/1.25 (C); preimmune serum, dilution 1/1.25 (D). Bar, 200 nm.

unaltered or enhanced attachment efficiency after treatment with neutralizing antisera has been described for adenovirus (29) and other virus systems, e.g., poliovirus (18) and influenza virus (30). The attached Ad2 virions treated with anti-Ad2 or antifiber serum did not become sensitive to DNase treatment to the same extent as the controls; this could possibly be explained by steric hindrance, leading to an inability of virions within complexes to properly reach the plasma membrane and thereby avoiding the cell-mediated destabilization process. Antifiber-treated virions remained on the cell surface (85%) or were located inside vesicles (15%). This cellular localization is in good agreement with the observations obtained by Brown and Burlingham (3), who used KB cells and an anti-Ad2 serum. From the results above, it is possible to suggest that the reduction in virus yield in the immunotitration assays when virions were incubated with antifiber-containing antisera is not just a reflection of aggregation but is also dependent on a neutralizing mechanism apart from a mere aggregation.

When the data from antihexon and anti-penton base neutralization were analyzed, it was found that the percentage of neutralization was always higher than the percentage of aggregated virions. Virions incubated with either of these antisera attached to cells and became sensitive to DNase treatment to the same extent as the control. It was previously shown that glutaraldehyde-stabilized virions become internalized but are unable to leave an endocytotic vesicle (36). When antihexon and anti-penton base sera were used at neutralizing concentrations, the majority of the virions were internalized but confined within vesicles. In the poliovirus and influenza virus systems, it has been demonstrated that neutralized virions not only attach to cells but also penetrate the cell membrane (19, 30), and in the case of the influenza virus, uncoating also occurs (30). The interaction between the penton base and the endosome membrane has been suggested to be important during adenovirus entry from an acidic vesicle into the cytoplasmic compartment (33, 34, 36). Possibly this interaction was blocked as a result of the reaction between virus and anti-penton base antibodies. However, 25% of the virions incubated with this antiserum succeeded in reaching the nuclear membrane. This agrees well with the result that the maximum level of anti-penton base neutralization of adenovirus never exceeded 45%. These observations also support the earlier proposal that not all virions appear dependent on an acidic vesicle and the penton base for a proper entry into the cytoplasmic compartment (36). This entry pathway is at present uncharacterized; however, an alternative entry mechanism of direct penetration has previously been described (3, 23).

It is obvious that when the three monospecific neutralizing antisera were used, the cellular distribution of neutralized virions was not the same; thus, an antifiber serum generally inhibited the relative virus internalization, and the antihexon and anti-penton base sera prevented the entry of virions from acidic vesicles into the cytoplasmic compartment. Besides the mere neutralization data presented in this communication, topological alteration of virions by attachment of different classes of antibodies could be a valuable tool in further studying the viral penetration and uncoating processes.

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IV.

NEUTRALIZATION OF ADENOVIRUSES:
KINETICS AND STOICHIOMETRY

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ABSTRACT

Antihexon-mediated neutralization of adenoviruses showed an immediate decline of surviving virus with time and the rate of neutralization was dependent on both concentration of antibodies and on temperature. The association between antibodies and virions was rapid and at high concentrations of antibody, a maximal degree of neutralization was achieved within one to two minutes after mixing of the reactants. Multiplicity curves performed with an antihexon serum displayed a linear correlation between the degree of neutralization and dilution of antiserum. Neutralization values experimentally obtained under steady state conditions, fully fitted a single-hit model based on Poisson-calculations. Only a fraction of the hexon-specific antibodies within a monospecific polyclonal antihexon serum was able to directly neutralize virus infectivity. However,other antibodies of the same serum were able to sensitize virions, which subsequently were neutralized upon addition of anti-rabbit IgG antibodies. Virions already attached to HeLa cells at $4^{o}C$ were to a large extent neutralizable by an antihexon serum, whereas antifiber and anti-penton base sera were negative. It is suggested that the major neutralizing mechanism displayed by an antifiber serum is through aggregation of the virions. An anti-penton base serum may neutralize the fraction of virus that enters the cells via an endocytotic mechanism; this is probably by a stoichiometric covering of the penton bases. Anti-penton base neutralization therefore contains components relevant for a multi-hit mechanism. Further aspects of the antihexon-mediated neutralization is discussed.

INTRODUCTION

In the adenovirus system the initial combination between virus and antibodies is a very rapid process and is completed within minutes (21). Such virus-antibody complexes are stable and no evidence for reversibility has been demonstrated (21). The kinetic curve of adenovirus neutralization, performed with a total anti-Ad 5 serum, is biphasic, starting with an exponential decline in viral survival followed by a decreased rate of neutralization. If antibodies are available in excess over the virus particles, no persistent fraction is detected (21,49). Only a certain fraction of the antibodies within an antiserum is able to neutralize virions (51). However, non-neutralizing antibodies are able to combine with virions, which are neutralizable upon subsequent addition of anti-IgG antibodies (22,48). Such virions are referred to as being sensitized as proposed by Notkins et al. (32).

The initial uncoating of adenoviruses takes place already at the cell surface and can be detected by an increased sensitivity of the viral genome to DNase treatment (44).

In a previous report on the interaction between HeLa cells and adenovirus neutralized by various antisera it was shown that anti-hexon- and anti-penton base-neutralized virions attach to cells to the same extent as untreated control viruses (54). Moreover, the neutralized virions become destabilized on the cell surface and penetrate the plasma membrane. Control virions are found free in the cytoplasmic compartment, whereas antibody treated virions are trapped within intracellular vesicles. Antifiber antibodies efficiently aggregate virions and such aggregates are able to attach to cells. Of the attached antifiber-treated virions around 15% is subsequently found in intra-cellular vesicles (54).

The aim of the present investigation was to analyze the neutralization of adenovirus with regard to kinetics and the effect of multiplicity of antibodies in different neutralizing systems. To further cast some light on the various mechanisms behind neutralization, the ability of various antisera to neutralize virions already attached to HeLa cells was examined.

MATERIALS AND METHODS

Cells and viruses: HeLa cells were maintained in suspension cultures at densities between 2.5×10^5 to 5×10^5 cells per ml in Eagle minimal essential medium (S-MEM) supplemented with 5% fetal calf serum and 5 µg of gentamicin per ml. Adenovirus type 2 was propagated and purified as previously described (12, 13).

Production of monospecific antisera: The soluble antigens remaining after virus isolation by preparative ultracentrifugation on CsCl gradients were separated by DEAE-cellulose chromatography as previously described (52). The hexon antigen was further purified by gel exclusion chromatography on Sepharose 6B and used in line immunoelectrophoresis (23). Immunoelectrophoretic lines formed between the hexon antigen and a total anti-Ad2 serum in agarose gels were cut out and compressed into dry films. One ml of phosphate-buffered saline (PBS) was added to the dried precipitate-containing agarose films which thereafter were ultrasonically treated and injected intramuscularily into a rabbit. Three immunizations were performed within a 4-week period (17) according to the procedure described above. Serum was collected eight days after the last immunization and further weekly for a period of one month. The material for each immunization contained approx. 3 µg of hexon antigen. The antihexon serum was heat inactivated at 56°C for 30 min before use. The production of antifiber-, anti-penton base-, and anti-Ad2 serum was described in a previous report (54).

Isolation of immunoglobulins from an antihexon serum: The virion-specific immunoglobulins within an antihexon serum were isolated by affinity chromatography on immobilized adenovirion-AH-Sepharose 4B

prepared as previously described (45). Portions (1 ml) of the anti-hexon serum were passed through the column in PBS containing 0.2 M NaCl. The retained material was eluted by a 0.2 M glycine-HCl buffer, pH 3.5 containing 0.2 M NaCl. All UV-absorbing material above 0.1 at 280 nm was dialyzed against bi-distilled water. After lyophilization, the immunoglobulins were resuspended in 1 ml of PBS.

Neutralization assay: The quantitative determination of the neutralization of virus infectivity was performed by a progeny virus immunotitration method as previously described (53). Briefly, 3×10^{10} virions in volumes of 25 µl were added to equal volumes of appropriate antisera or immunoglobulin-fractions and when necessary, these were diluted in PBS containing 1% of bovine serum albumin (BSA) to yield final serum or immunoglobulin dilutions ranging from 1/2 to 1/4,000. In some instances 100 µl or 250 µl of serum were added to 25 µl of virus and these series will be referred to as the 1/1.25 and 1/1.1 samples, respectively. After incubation at 37°C for 30 min, PBS containing 1% BSA was added to each sample to give a final volume of 1,550 µl. Samples of 25 µl from such virus-antibody mixtures were removed and added to 3 ml of S-MEM containing 1.52×10^7 cells, yielding a multiplicity of infection (moi) of 32 particles per cell, being equivalent to one infectious unit per cell. The cells were incubated at 37°C in suspension culture and at 39 h post infection (p.i.) progeny virus was quantitatively isolated by a one step CsCl gradient centrifugation procedure. The progeny virus was disrupted in the presence of 5M urea and subsequently quantified by rocket immunoelectrophoreses against an antihexon serum in a standardized system. Neutralized virus samples were compared with a separate control in which the neutralizing agent was replaced by PBS containing

1% BSA. The working range in the standardized assay was 0-94% of neutralization due to the detection limit of progeny virus when few cells were infected. For this reason maximal neutralization was manifested as $\geq$ 94%.

Kinetic analyses: Prewarmed virions in volumes and quantities as described above, were mixed with appropriate and likewise prewarmed antisera diluted to yield either around 50% or $\geq$ 94% neutralization. The mixtures were incubated at 37°C for 0 to 60 min. At indicated times, cold PBS containing 1% BSA were added to the mixtures to yield final volumes of 1,550 µl. Samples of 25 µl were immediately withdrawn and added to 1.52×10^7 cells. Further incubations and assay of progeny virus production was as described above.

Neutralization of virions attached to HeLa cells: Virions were added to HeLa cells at a multiplicity of 100 particles per cell and the virus-cell mixtures were incubated at 4°C for 45 min. Under these conditions a final attachment of around 30 virus particles per cell was anticipated (33). Unattached virus was removed by centrifugation and the cell suspension was divided into several flasks at each experiment, yielding 1.52×10^7 infected cells per bottle in a total volume of 1 ml. Antisera, as indicated in the text, were added to each virus-cell mixture and the mixtures were incubated for another 30 min at 4°C, whereafter these were transferred to 37°C. After incubation for 30 min, 34 ml of medium was added to the cells and at 39 h p.i. the samples were analyzed for progeny virus production as described above.

Chemicals: Eagle minimal essential medium, fetal calf serum and gentamicin were obtained from Flow Laboratories Ltd., Irvine, Scotland. Agarose (type I) and bovine serum albumin were from Sigma Chemical Co., St. Louis, Mo. AH-Sepharose 4B and Sepharose 6B were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Anti-rabbit IgG serum was obtained from Cappel, Cochranville, PA.

RESULTS

Neutralization of virions by different antisera: Heat-inactivated antisera at final dilutions ranging from 1/1.1 to 1/4,000 were mixed with a constant amount of virions and incubated for 30 min at 37°C. The extent of virus neutralization was analyzed according to the progeny virus immunotitration method as described in Materials and Methods. A final antihexon serum dilution of 1/2 displayed a neutralization of virus infectivity of $\geq$ 94% (Fig. 1). At serum dilutions between 1/8 to 1/200 the extent of neutralization gradually decreased. An antifiber serum displayed a virus neutralization of $\geq$ 94% at a final dilution of 1/200 and upon further serum dilutions the degree of neutralization decreased. As also was reported in a previous investigation (54), the anti-penton base serum caused a maximum of around 50% of neutralization at final dilutions of 1/1.1 and 1/1.25. If the concentration of the anti-penton base serum was increased six times as compared with the 1/1.1 sample, no further increase in the degree of neutralization was observed.

Kinetic analyses of virus neutralization: An antihexon serum at the two final dilutions of 1/1.25 and 1/20, yielding $\geq$ 94% and a 50% level of neutralization, respectively, (Fig. 1) was analyzed regarding the kinetics of neutralization. Virions were mixed with either of the two serum-dilutions and incubated for periods between 0 and 60 min, whereafter samples were analyzed for surviving virus according to the immunotitration method. For the high antihexon serum concentration, plotting of the survival data against time revealed a straight line (curve 1, Fig. 2A), i.e. a constant fraction of virus was neutralized per unit of time. After 1-2 min, a maximal degree of neutralization was reached and maintained upon

prolonged incubation. Curve 2 in Fig. 2A (dil. 1/20) followed a linear or curvi-linear course for approximately 3 min, whereafter the rate of neutralization levelled off to finally reach the 50% plateau of survival. An interesting feature of this curve was the fact that even after 60 min of incubation no steady state was reached and obviously a continuous process of neutralization still occurred. Both curves were indicative of a single-hit mechanism since neither of them was preceeded by a lag period before the commencement of the exponential decrease of infectivity. But, the possibility exists that the association between viruses and antibodies was too rapid to be measured accurately by this technique, and therefore a kinetic experiment of the 1/20 dilution of the antihexon serum was performed at 4°C. The initial rate of neutralization appeared to be lower at this temperature, but the final level of neutralization reached, after prolonged incubation, was almost the same as at 37°C (Fig. 2A and B). No lag period appeared at the lower temperature. An antihexon serum at a final dilution of 1/30, thus yielding around 35% of neutralization (Fig. 1), did not reveal any lag period when tested in the same way at 37°C (data not shown).

Antibody-virion ratio required for neutralization: The kinetic curves indicated that one virion was neutralized by a single antibody in the case of antihexon neutralization. To further test this hypothesis, an antihexon serum at various dilutions was analyzed for its ability to neutralize virions. Since steady state values were not reached after 60 min of incubation as shown above, the virus-antibody mixtures were incubated at 37°C for 3 h and further at 4°C for 21 h, whereafter the remaining virus infectivity was determined according to the immunotitration method.

Repeated determinations of the extent of virus neutralization caused by an antihexon serum at a final dilution of 1/10 revealed a mean survival value of 13.6% with a coefficient of variation of 4%. Based on this figure of the survival level, the multiplicity of antibodies to virions was calculated according to the Poissonian distribution by assuming a single-hit or a two-hit model (Table 1). Theoretical survival values were subsequently calculated for various antihexon serum dilutions. The single-hit and two-hit survival values for antihexon neutralization at a final dilution of 1/20 was 36.9% and 47.8%, respectively, which should be compared with the corresponding experimental value of 38.1%. At higher serum-dilutions the discrepancies between the two models became more evident, e.g. at a final dilution of 1/75, the theoretical values for single-hit and two-hit neutralization models were 76.6% and 92%, respectively, with the former figure approaching the experimentally obtained value of 73.3%. As outlined in Table 1 it is obvious that the experimentally obtained values closely fit the theoretical values for a single-hit model, which is demonstrated graphically in Fig. 3.

An equation for the experimental data revealed a straight line originating at lg 2.01 with a regression coefficient of 0.998.

Involvement of accessory factors in the neutralization: The involvement of thermolabile components within the antihexon serum or in an anti-Ad2 serum was examined. Heat-inactivated and untreated antisera, both at different dilutions, were run in parallel in the immunotitration assay. No differences in the ability to neutralize was found between heated and unheated antisera, indicating that no thermolabile components were crucial for an enhancement of neutral-

ization of adenovirus by antihexon or anti-Ad2 antibodies.

Neutralization by the immunoglobulin fraction from an antihexon serum and sensitization of virions: The antibody-fraction of an antihexon serum was isolated by affinity chromatography on an immobilized adenovirion-matrix as described in Materials and Methods. Geldiffusion between the bound material and an anti-rabbit IgG serum produced distinct precipitates (data not shown). The isolated immunoglobulin fraction was tested for its ability to neutralize virus infectivity. At a final dilution of 1/2 the immunoglobulins displayed a neutralization of $\geq$ 94%. At higher dilutions a reduction in the neutralizing power was observed, and compared with the antihexon serum (Fig. 1) the ability of the immunoglobulins to neutralize virions was somewhat lowered. A possible reason for this could be that only UV-absorbing material above 0.1 was collected when the immunoglobulins were isolated on the adenovirion-matrix.

To investigate whether only a fraction of the antihexon antibodies within an antihexon serum was able to neutralize virus infectivity, the following experiment was carried out. An antihexon serum at a dilution of 1/20, yielding around 50% of neutralization, was mixed with virions at 37^{o}C. After incubation for 30 min, anti-rabbit IgG serum was added and the mixture was incubated for another 30 min, whereafter the surviving virus was quantified according to the immunotitration method. It was demonstrated that the degree of neutralization was enhanced to $\geq$ 94% after addition of anti-rabbit IgG antibodies. The immediate interpretation of these results is that antibodies with different hexon-specificities exist within a polyclonal antihexon serum and only a fraction of these antibodies

is able to directly neutralize virions.

The mechanism of neutralization by addition of anti-rabbit IgG-antibodies is probably through aggregation of virions, since 96% of the virions in this reaction mixture was found on a CsCl-cushion upon sucrose-gradient centrifugation on 10-25% sucrose, performed as previously described (54). The same result as described above was obtained when the immunoglobulin fraction of the antihexon serum was analyzed in a similar way.

The antihexon serum used in this investigation was prepared in a rabbit without the aid of Freunds complete adjuvant (FCA). In a previous study on the interaction between neutralized virions and HeLa cells (54), an antihexon serum was prepared by immunizing soluble hexon-antigen together with FCA. Since it is well established that the use of FCA in the immunization procedure, yields antisera with a majority of high affinity antibodies (36) it was of interest to see whether the results above were due to a preferential production of low affinity antibodies in the antihexon serum used in the present investigation. For this purpose, an antihexon serum produced by immunization of soluble hexon and FCA was diluted to yield around a 20% level of neutralization. Upon addition of anti-rabbit IgG antibodies as described above, the degree of neutralization was enhanced to 90%, indicating that in both types of antihexon sera, only a fraction of the antibodies was able to directly neutralize the virions.

Neutralization of virions attached to HeLa cells: Virions were mixed with HeLa cells at $4^{o}C$, under which condition no penetration of virus occurs (43). After removal of unattached virions, different volumes

of various antisera were added to the virus-cell mixtures and incubations were continued for 30 min at 4°C, whereafter the mixtures were transferred to 37°C. After incubation for 30 min, S-MEM was added to the mixtures and at 39 h p.i. progeny virus production in each series was determined by the immunotitration method. As shown in Table 2, addition of an antifiber serum or an anti-penton base serum did not in any instance significantly influence the production of progeny virus as compared with control infections. However, an antihexon serum seriously affected the progeny virus production at all the dilutions tested.

DISCUSSION

An immediate decline of surviving virus in neutralization kinetic curves has often been taken as an indication that one antibody per virion is needed for a successful neutralization (8, 15, 26, 39). On the other hand, when a lag period is observed before the inactivation phase begins, this is often interpreted as the result of an involvement of several antibodies in the process of neutralizing one virion (29). Recently, this way of dealing with kinetic data has been challenged by Daniels (6) and Della-Porta and Westaway (7). In the view of these authors, the association between virions and antibodies is too rapid to be measurable by conventional techniques, and therefore the absence of a lag period does not necessarily imply that one antibody inactivates one virion. In the western equine encephalitis virus system, showing a single-hit mechanism at 37°C, a lag period is observed if the temperature is lowered to 4°C (5, 8), thus demonstrating the rapidness of the union between antibodies and virions at higher temperatures. Lafferty (24) showed that a dilution of antiserum was accompanied by a shift from single-hit to multi-hit curves in the influenza virus system. Thus it is desirable not to judge whether neutralization is a single-hit or a multi-hit phenomenon when based solely on kinetic studies. By constructing multiplicity curves, i.e. plotting the fraction of surviving virus infectivity versus antibody concentration, a straight line originating from the origin of the plot is often obtained (8, 15, 16, 26). Since the virus-antibody mixtures are incubated for an extended period of time, often for several hours or days, such steady state neutralization values would be preferable to deal with in judging whether the neutralization process obeys the order of a single-hit or a multi-hit model.

In this investigation, no lag period was observed in the kinetic curves even if the antihexon serum was highly diluted or if the temperature was lowered to 4°C, thus indicating a single-hit mechanism.

The observation that the survival value at time zero was 98% in the kinetic curve 2 (Fig. 2A), implies that at least the interval 98-0% of survival is within the working range of the methods used in these experiments, thus a significant lag period would have been detected. The zero value is actually a 30 second value if taken into account the time needed for addition of PBS containing 1% BSA to the virus-antibody mixture, mixing of the sample and transferring aliquots to HeLa cells.

Poisson calculations revealed that the steady state neutralization values fitted well with a single-hit model. However, this requires that the antibodies attach to the virus particles according to the Poissonian distribution. The validity of this assumption has been demonstrated in the poliovirus system, in which radioactively labeled antibodies attach to virions according to this distribution (18). A similar theoretical arguing was performed by Dulbecco et al. (8) in a study of poliovirus and western equine encephalitis virus neutralization and in which viral survival data were used for calculation of antibody equivalents attached to virions. However, when suggesting a single-hit mechanism for antihexon neutralization in the adenovirus system one must keep in mind the possibility that several different species of antihexon antibodies could be involved in this process and if one such class is limiting for neutralization, false single-hit values may be obtained.

The kinetic curves in Fig. 2 revealed a marked biphasic rate of neu-

tralization. In the first part of the kinetic curves an exponential decline in surviving virus was observed, followed by a second part with a decreased rate of neutralization. In other virus systems it has been proposed that the second part of such kinetic curves and the occurrence of a persistent fraction is due to interference of non-critical antibodies with critical neutralizing antibodies and the latter are thereby hindered from reaching the critical sites, leading to a lack of neutralization. Addition of anti-IgG antibodies to such virus-antibody mixtures neutralizes virions by covering the unreacted critical sites (16, 25, 32).

In the adenovirus system it seems as if non-critical antibodies may attach to virions and neutralization is achieved upon addition of anti-rabbit IgG antibodies (22, 48). This could be a reflection of aggregation, since virions were found on a CsCl-cushion upon sucrose-gradient centrifugation in the present investigation. Kjellén has shown that the second part of the kinetic curve is not due to combination of more antibodies to virions, but rather to reactions occurring within the already formed virus-antibody complex (21). Moreover, kinetic studies with neutralizing monoclonal antibodies reveal persistent fractions in various virus systems (19, 47). In such preparations, the antibodies are homogenous with regard to specificity and interfering non-critical antibodies are not available, yet a persistent fraction exists. It thus appears as if the occurrence of a persistent fraction is due to an inherent variation in binding specificity of the neutralizing antibodies, thus leading to incorrect combination of some antibodies to the virions. In the adenovirus system, the first part of the kinetic curve could represent a successful combination of antibodies to virions, thus directly

rendering the virus non-infectious. Due to incorrect combinations of some antibodies to virions, the rate of neutralization is retarded and this second part of the kinetic curve would then represent neutralization of infectious virus-antibody complexes due to rearrangements of the antibodies within these complexes.

No information about the mechanism of antihexon neutralization is at present available, but if one antihexon antibody neutralizes one virus particle, it is tempting to speculate about a conformational change of the viral capsid as one possible factor to consider. This model also fits well with the fact that an antihexon serum is able to neutralize virions already attached to HeLa cells. The conformational neutralization pathway has been demonstrated for a variety of virus systems such as poliovirus (9, 10, 27, 28), influenza virus (38) and bovine enterovirus (3).

Antisera against the fiber antigen have been reported both to contain (1, 20, 30, 50) and to lack (22, 34, 35, 48) significant amounts of neutralizing antibodies. Most probably this is a reflection of varying degrees of aggregation of viruses and employment of different assay systems. In vivo studies have shown that immunization with purified fiber protected experimental animals against the challenge of a lethal dose of adenovirus type 5 (30). In a study in humans it was demonstrated that fiber-immunized volunteers were almost totally protected against occular challenge with homologous adenovirus type 1, as compared with unimmunized control persons (20). Thus, even though several investigators have demonstrated that antifiber sera are devoid of neutralizing antibodies, the above mentioned experiments in vivo clearily show the protective role of the fiber as immunogen.

An antifiber serum efficiently aggregates virions (11, 46, 54) and working with a total anti-Ad2 serum, Smith et al. (42) showed that aggregation of virions was most pronounced at equivalent concentrations of antibodies and virions, and no aggregation was observed at high antibody concentrations. In most neutralization studies, small amounts of viruses are mixed with antibodies in great excess, under which conditions minor or no aggregation occurs and subsequently no neutralization is observed. In a previous study it was shown that the degree of aggregation mirrors the neutralization data (54). Attempts in this study to avoid aggregation of virions by addition of a great excess of antifiber antibodies were not successful. Most probably this is a reflection of the large amount of virions used in the immunotitration assay (data not shown).

It has been reported that an antifiber serum neutralizes virions when measured by the FFU-assay but not when analyzed by the plaque-reduction assay (34). Usually the final readings are made at 48 h in the FFU-assay and at around two weeks in the plaque-titration assay, which could imply that the results obtained with the FFU-assay include a delayed start of the multiplication process due to interference of antifiber antibodies with e.g. viral penetration into the cells. Upon prolonged incubation periods the results obtained with the FFU-assay did not differ from those of the plaque-assay (37). In the progeny virus immunotitration assay, the hexon antigen production in the infected cells is analyzed as a technical control together with the progeny virus production. When antifiber neutralized virions were incubated for a prolonged period of 4 days instead of the normal 39 h, no progeny virus or hexon antigen production was initiated.

It was previously shown that antifiber neutralized virions attached to HeLa cells and around 15% of the attached virions were able to penetrate the cell membrane and were found in intracellular vesicles and usually in aggregates of 10-20 particles (54). This implies that although some aggregated virus particles are able to penetrate the cell membrane, these virions are not able to initiate replication probably because proper uncoating can not occur due to the aggregational status.

It appears as if adenovirus may enter the cell by two alternative mechanisms. One is by direct penetration of the plasma membrane (2, 31), and the other is by an endocytotic process (4, 14, 43). The penton base has been shown to play a crucial role for a proper virion entry from the endosomes into the cytoplasmic compartment (40, 41, 43). Since the maximal level of anti-penton base neutralization was around 50% irrespective of antibody concentration, it was in a previous study speculated that the endocytotic penetration pathway was inhibited by anti-penton base antibodies (54). A new anti-penton base serum has recently been prepared by immunizing rabbits with immunoelectroprecipitates. This antiserum also revealed a maximal level of neutralization of 50% (data not shown). This implies that the neutralizing effect of the anti-penton base sera is not due to contaminating antifiber or antihexon antibodies. The fact that virions already attached to HeLa cells are not neutralizable by an anti-penton base serum may indicate that these antibodies are not able to associate with sufficient numbers of penton bases on the viral surface once the virion is attached to a cell. This multi-hit hypothesis for anti-penton base neutralization is further strengthened by kinetic studies, which in some instances showed a marked tendency of a lag period before the decline in

survival, thus indicating that several antibodies are involved in anti-penton base-mediated neutralization (data not shown). It thus appears as if anti-penton base antibodies play a stoichiometrical role in the neutralization process and several, if not all, of the penton bases have to be covered with antibodies for achievement of full neutralization of the "endosome-dependent" virions.

To summarize, it seems as if a variety of neutralizing mechanisms exist within the adenovirus system. An antihexon serum neutralizes virions probably via a single-hit mechanism. An anti-penton base serum neutralizes the endosome-dependent virions by stoichiometrical covering of the penton bases and thus obeys a multi-hit model. An antifiber serum aggregates virions and a minority of these virions are transported into the cells but no initiation of replication occurs. Further studies on these neutralizing processes will be performed using monoclonal antibodies which are under preparation.

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FIGURE LEGENDS

Figure 1: Neutralization of virus by different antisera. Virions were mixed with different antisera and assayed for neutralization by the progeny virus immunotitration method. Neutralization is expressed as the percentage of reduction in progeny virus yield as compared with that of untreated control infections. ●, antifiber serum; ▲, antihexon serum; ■, anti-penton base serum. Note the change in scales on the abscissa.

Figure 2. Kinetic curves of the antihexon neutralization of adenovirus. A. Constant amounts of an antihexon serum at final dilutions of 1/1.25 (curve 1) or 1/20 (curve 2) were mixed with virions and incubated for indicated periods of time at 37°C, whereafter surviving virus was determined according to the immunotitration method.
B. An antihexon serum at a final dilution of 1/20 was mixed with virions and incubated at 4°C, and further processed as described above.

Figure 3. Steady state neutralization with increasing antibody concentrations. A logarithmic transformation of surviving data of Table 1 is plotted against the relative serum concentration. A and B are theoretical representations of the curves for two- and single-hit models, respectively. The filled circles represent the experimental data.

Table 1

Theoretically and experimentally obtained neutralization values at various antihexon serum dilutions.

Final dilution of an antihexon serum	% SURVIVING VIRUS		
	Experimental values	Theoretical values[a]	
		Single-hit model	Two-hit model
1/8	8.8	8.3	6.8
1/10	13.6	13.6	13.6
1/15	27.2	26.4	32.3
1/20	38.1	36.9	47.8
1/30	51.4	51.4	67.5
1/40	62.1	60.7	78.2
1/50	72.9	67.1	84.4
1/75	73.3	76.6	92.0
1/100	89.2	81.9	95.1

[a] The Poisson calculations were based on serum dilution 1/10 and according to $e^{-\lambda} = 0.136$ and $e^{-\lambda} + \lambda \cdot e^{-\lambda} = 0.136$ for determinations of the multiplicity of antibodies (λ) needed to yield a surviving fraction of 0.136 in the single-hit and two-hit models, respectively.
The amount of antibodies in the different serum dilutions was then calculated based on that in the 1/10 dilution and the corresponding survival value was obtained from the formulas above.

Table 2

Neutralization of virions attached to HeLa cells.

	µl antiserum added to the virus-cell-mixture[a]			
Antiserum-treatment	500	250	50	25
Antihexon	≥ 94[b]	≥ 94	82	77
Anti-penton base	0	2	15	13
Antifiber	13	8	8	8
Preimmune	NT[c]	0	0	NT

[a] Around 30 virus particles were attached to each cell at 4°C, and further incubations were as described in Materials and Methods

[b] The figures refer to the percentage of neutralization

[c] Not tested

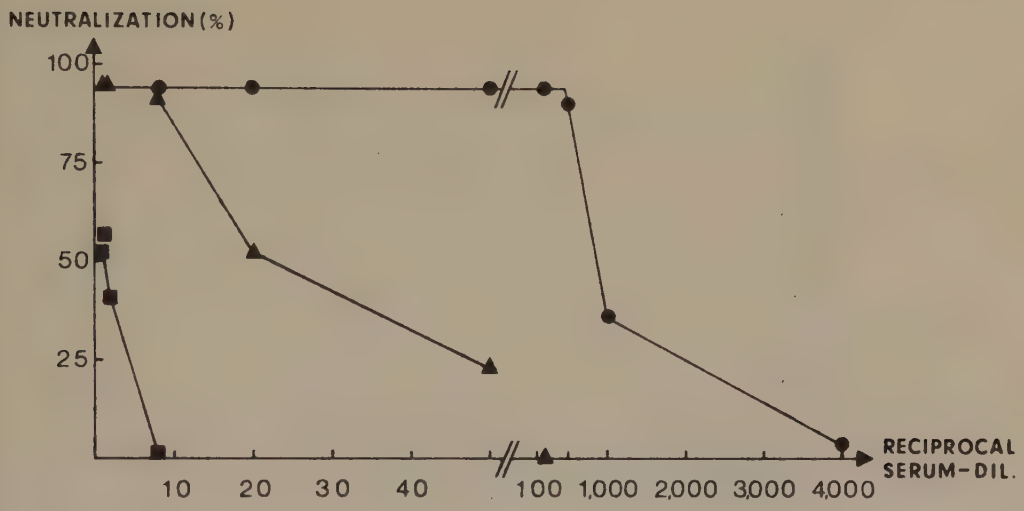
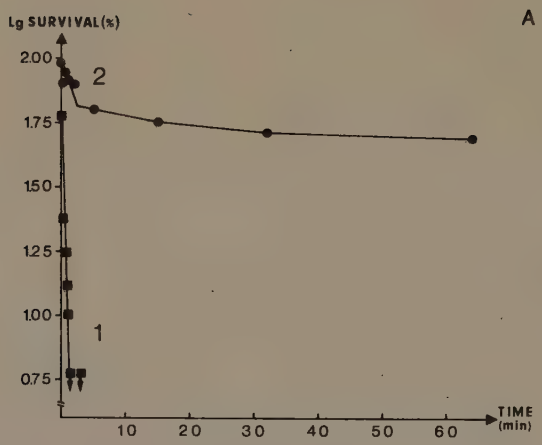

NEUTRALIZATION (%)
100
75
50
25
10
20
30
40
100
1,000
2,000
3,000
4,000
RECIPROCAL
SERUM-DIL.

Figure 1


A
Lg SURVIVAL(%)
2.00
1.75
1.50
1.25
1.00
0.75
2
1
10
20
30
40
50
60
TIME
(min)

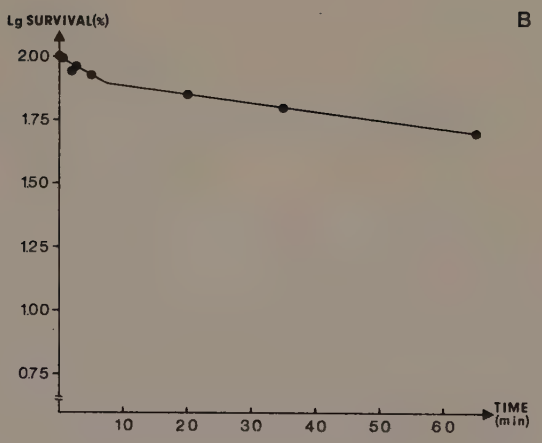

B
Lg SURVIVAL(%)
2.00
1.75
1.50
1.25
1.00
0.75
10
20
30
40
50
60
TIME
(min)

Figure 2

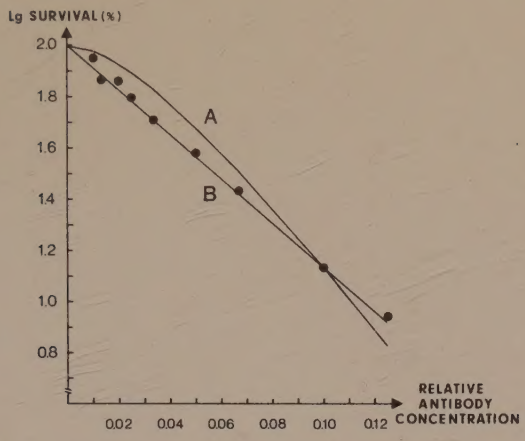

Lg SURVIVAL (%)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
A
B
0.02
0.04
0.06
0.08
0.10
0.12
RELATIVE ANTIBODY CONCENTRATION

Figure 3